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The Effects of Propofol, Sodium Pentobarbital, and Ketamine Hydrochloride on *In Vitro* Mouse Embryonic Development

Tyler C. Balak
Old Dominion University

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by

Tyler C. Balak
B.S. Biology, 2004, University of North Carolina at Wilmington

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Approved by:

R. James Swanson (Director)

Christopher Osgood (Member)

Lesley Greene (Member)

ABSTRACT

THE EFFECTS OF PROPOFOL, SODIUM PENTOBARBITAL, and KETAMINE HYDROCHLORIDE ON *In Vitro* MOUSE EMBRYONIC DEVELOPMENT

Tyler Cameron Balak
Old Dominion University, 2015
Director: Dr. R. James Swanson

Every year more than 75,000 pregnant women are exposed to teratogenic medications or general anesthesia during non-obstetric surgery in the US,[1-4] and embryonic effects of general anesthesia are of particular interest in laboratory research and veterinary medicine. The mouse system is used to screen potential toxic effects of anesthetics used in egg retrieval for in vitro fertilization (IVF), or those of pharmacologic agents which may come in contact with the egg or early embryo. Mouse preimplantation 2-cell embryos were exposed in vitro to incremental concentrations of common general anesthetics within and exceeding the normal clinical dosage range for mice (propofol 50-200mg/kg, sodium pentobarbital 50mg/kg, and ketamine hydrochloride 50-100mg/kg) for 72h to determine the effects of each anesthetic on subsequent cleavage and development during prolonged exposure. The anesthetic dosage ranges were as follows: propofol 0-25.0μg/mL, sodium pentobarbital 0-300μg/mL, and ketamine hydrochloride 0-5000μg/mL, embryonic development was evaluated every 24h during the 72h mouse embryo assay culture period. Results found propofol (≥ 2.0μg/mL), sodium pentobarbital (≥30μg/mL), and ketamine hydrochloride (≥40μg/mL) to exert inhibitory effects to the in vitro development of preimplantation mouse embryos. The percentage of 2-cell embryos developing to more advanced stages was decreased by exposure to higher concentrations of all three compounds. By the 72h endpoint, blastomeres of delayed and arrested embryos began to degenerate, displaying lysis and/or fragmentation; embryos exposed to lower concentrations of each anesthetic demonstrated a significant ability to continue cleavage and differentiation towards blastocyst formation. Conclusion: Embryotoxic effects to preimplantation embryos were observed in a dose-dependent manner after 72h of in vitro culture with propofol, sodium pentobarbital, and ketamine hydrochloride.

This dissertation is dedicated to the philosophy that "Wise men argue causes; fools decide them."

-Anacharsis

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CHAPTER 1

INTRODUCTION

Potentially teratogenic medications are prescribed to millions of women of childbearing age (age 14-44) in the US each year[1] and approximately 0.5-2% of pregnant women are exposed to general anesthesia during non-obstetric surgery in the United States each year.[2-4] For some women, surgery is performed before detection of pregnancy, for others surgery may be necessary in spite of the pregnancy.[3,5] Unavoidable surgery may be directly related to a patient's pregnancy, such as in the case of cervical cerclage placement, or indirectly related to a patient's pregnancy, such as ovarian cystectomy; but may also include completely unrelated emergency procedures such as appendectomy, biliary tract disease, cardiac disease, cancers, or treatment of injuries such as bone fracture.[3,5,6]

The use of potentially teratogenic medication is sometimes necessary, since for some applications effective nonteratogenic medications do not exist.[1] The teratogenic risk of anesthetics in humans is difficult to assess,[5] however numerous studies suggest exposure to anesthesia may have potential detrimental effects on development.[6-16] In addition to utilization for typical non-obstetric surgical procedures,[2-4] anesthesia is necessary for assisted reproduction procedures such as in vitro fertilization (IVF) and gamete intrafallopian transfer.[13] Ideally, if a surgical procedure must be performed during pregnancy, it should occur during the second trimester to avoid the period of organogenesis during the first trimester, and to minimize the risk of inducing preterm labor during the third trimester.[17] Adequate information is not available to definitively ascertain which anesthetics used for these procedures could be toxic to the oocyte or the developing embryo,[13] making the study of the effects of anesthetics to early embryonic development an area of particular interest.

Anesthesia is defined as the absence of sensation, contrasting with analgesia, the relief of pain.[18] Anesthesia may be classified as either local/regional (in a specific area of the body), or general (absence of sensation in the entire body).[18] The general anesthesia state is a balance of numerous effects including unconsciousness, analgesia, immobility, and suppression of reflexes and stress response.[19] The four stages of general anesthesia consist of induction, maintenance, emergence, and recovery.[20] Induction of general anesthesia involves the safe and rapid passage of a patient from the conscious to the unconscious state.[20] Induction may be accomplished by intravenous (IV), inhalational, and less commonly intramuscular (IM), or intraosseous (IO) means, to achieve adequate concentrations of anesthetic agent within the brain.[20]

History Of Anesthesia

The father of chemistry and founder of medicinal chemistry and toxicology, Renaissance physician Paracelsus anticipated the practice of anesthesia with his philosophy that "no thing is without poison; solely the dose determines that a thing is not a poison.[21,22]" Until the discovery of anesthesia, surgical patients

were fully conscious during what can only be described as quick and brutal procedures.[23] Throughout history, numerous agents have been used for hypnotic or sedative properties, such as alcohol, opium, and narcotic plants such as hemp, jimsomweed, belladonna, henbane,[24] and coca.[23]

Modern anesthesia emerged with the discovery of the effects of two gases, diethyl ether and chloroform.[21,23] Boston dentist William Morton, the founder of modern anesthesia, investigated diethyl ether inhalation after discovering that significant anesthesia was produced by topical application of ether to the teeth of a patient teeth before extraction.[23] After great success with inhalation of volatile ether for unconscious, painless tooth extraction, in 1846 Morton assisted the Chief of Surgery at Harvard, John Collins Warren, in performing the first surgery using general anesthesia.[23] Concurrently, Scottish obstetrician James Young Simpson was determined to find a better anesthetic than ether, and once introduced to chloroform, he was inspired to promote its use as a volatile anesthetic.[23] Chloroform anesthesia became widely accepted and its use remained prevalent for decades;[23] chloroform was so popular that it was used anesthetize Queen Victoria for the birth of Prince Leopold in 1853.[25]

During the development of anesthesia in the late 19th and early 20th centuries, several hundred anesthetic gases were discovered and used, however almost all were be explosive (except chloroform and nitrous oxide),[23] and inhalation of these gases often resulted in slow, unpleasant, and occasionally dangerous inductions.[21] The discovery that fluorination of volatile gases reduced its flammability led to the development of superior volatile anesthetics.[23] Volatile agents such as isofluorane and sevoflurane continue to be used extensively as general anesthetics,[20,23] and desflurane or halothane may also be used occasionally.[20]

Although anesthesia transformed modern medicine, the necessary inhalation of vapors seemed unpleasant to many patients,[23] as well as sometimes causing slow or dangerous inductions.[21] Little alternative to inhalation anesthesia was available prior to development of the IV route, following the mid-19th century technological advancement of hollow needles and syringes.[19,21] Drug administration through direct access to the bloodstream allowed the rapid induction and maintenance of general anesthesia.[19,21] The first IV anesthetic successfully used was chloral hydrate in 1872.[23,24,26] Various other intravenous drugs were experimented with, but most were abandoned once side effects became apparent,[23] and IV anesthesia did not become popular until the 1930s with the clinical introduction of barbiturates.[26]

Originally synthesized by Volwiler and Tabern in 1930,[24] pentobarbital and its sulfur derivative thiopental revolutionized intravenous anesthesia.[23,24] John Lundy and colleagues of the Mayo Clinic introduced pentobarbital and thiopental anesthesia for clinical use, and barbiturates use as IV anesthetics quickly gained popularity.[23, 24] During this time, many barbiturates were developed, however the only one to offer any significant challenge to thiopental and pentobarbital was the ultrashort-acting hexobarbital.[21,27] Hexobarbital use was limited due to the fact that induction was not as smooth[27] and it allowed some myoclonic movement.[21,24] Derivation of thiopental by the addition of a sulfur group to pentobarbital increased the lipid solubility to alleviate problems caused by slower acting pentobarbital,[24] and thiopental

lacked excitatory movements manifested by hexobarbital.[21] Barbiturates had many undesirable properties though, including cardiovascular depressant action,[23] instability of solutions, irritant effects at the site injection, absence of analgesia, and unfavorable pharmacokinetics for extended use,[27] ultimately contributing to the development of new induction agents.[21,27]

The first non-barbiturate IV anesthetics such as dolitrone and hydroxydione had undesirable properties as well.[27] The development of etomidate in 1974[27] demonstrated an agent which had several benefits over barbiturates, including minimal cardiovascular depressant effects.[23,27] In addition, several of its undesirable effects were minimized with prior use of fentanyl, for "balanced anesthesia."[27] Balanced anesthesia, a concept first theorized by Lundy in 1926, uses a combination of premedication, local anesthetic, and general anesthetic to reduce the dose of each agent to minimize side effects and improve safety.[21,23]

Ketamine (Ketalar) was synthesized in 1962[23,28] and was quickly adopted by the American military during the Vietnam War for its favorable hemodynamic effects, and was later released for civilian use.[29] At the time, ketamine was believed to be the perfect anesthetic because it provided loss of consciousness and antegrade amnesia[28] as well as good analgesia even at subanesthetic concentrations,[30] however emergence from ketamine anesthesia produced undesirable psychotomimetic side effects,[31,32] leading to the continued search for an ideal anesthetic. Later work with ketamine showed the retention of desirable and absence of undesirable properties of the pharmacologically available racemic mixture with use of enantiomers of ketamine; however the introduction of other agents such as propofol overshadowed these potential benefits.[27] Propofol (Diprivan) was clinically introduced in 1977 as a highly lipophilic short-acting IV anesthetic.[15,23,33-43] Compared to other anesthetics, propofol is advantageous due to its rapid action with short, predictable duration, and rapid, antiemetic, clear-headed recovery, making propofol arguably the most frequently used IV induction agent.[15,23,30,33-43]

Anesthetic General Information

General anesthetics are highly selective, only binding to a small number of targets in the central nervous system (CNS).[44] Drug transport to vessel-rich tissues such as the brain is necessary for rapid onset of action, and IV administration allows immediate introduction of a dose to the bloodstream.[45] The primary targets of IV anesthetics are CNS ion channel-linked receptors for the either the endogenous neurotransmitter glutamate, the main excitatory neurotransmitter in mammals, or gamma-aminobutyric acid (GABA),[21] the most abundant inhibitory neurotransmitter.[19,21,46-50] GABA is synthesized by glutamic acid decarboxylase from L-glutamic acid,[51] and GABA modulates inhibition in the CNS by activating two classes of receptors, ionotropic ($GABA_A$ /$GABA_C$) and metabotropic ($GABA_B$).[47,50,52,53] Metabotropic $GABA_B$ receptors couple to G proteins (guanine-nucleotide-binding proteins) to influence the movement of potassium and calcium ions and modulate synaptic transmission through intracellular effector systems.[52-55] Ionotropic $GABA_A$ receptors are ligand-gated ion channels that convey fast synaptic transmission,[53] and

most IV anesthetics (except ketamine) exert their primary action by enhancing inhibitory signaling at $GABA_A$ receptors.[19,21,44]

The $GABA_A$ receptor is a pentameric transmembrane glycoprotein of about 275 kDa[47] composed of phylogenetically related subunits (α1-6, β1-4, γ1-3, δ, ε, ρ1-3)[56] assembled around an integral transmembrane chloride (Cl^-) channel.[19,47,56] GABA facilitates inhibition[47,50] by binding to $GABA_A$ receptors, regulating the gating of the Cl^- channels[47,48,57] to hyperpolarize neuron membranes.[21] In addition to the GABA recognition site, the $GABA_A$ receptor includes a variety of allosteric modulatory sites through which many pharmacological agents can influence receptor function[58] by enhancing the binding of GABA, while inhibiting the binding of GABA antagonists.[50,58,59] The $GABA_A$ receptor is the primary target of a diverse group of anxiolytic, sedative, anticonvulsant, and anesthetic pharmacological agents, including barbiturates, benzodiazepines (midazolam, valium, diazepam, clonazepam), ethanol, neurosteroids (pregnenolone, dehydroepiandrosterone), and general anesthetics such as halothane and 2-6-diisopropylphenol (propofol).[48,50,58-60]

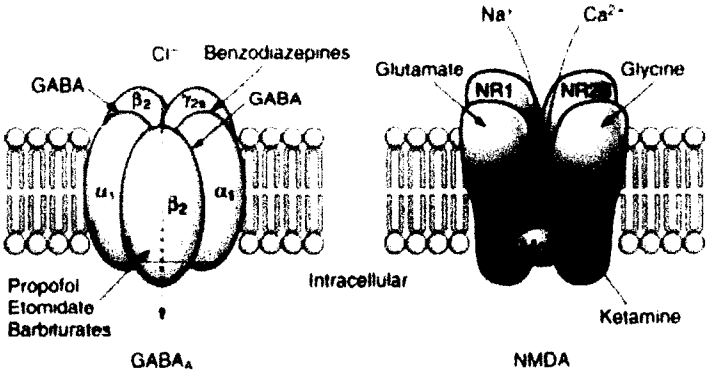

Fig. 1. Key targets of intravenous anesthetics. $GABA_A$ receptors are critical targets for benzodiazepines, barbiturates, etomidate, and propofol. Although it is possible for the drugs and ligands to interact with this protein in multiple areas, it is generally agreed that the endogenous ligand GABA binds to the receptor in a pocket between the α and β subunits. Many of the intravenous anesthetics have their main influence on the activity of this protein in the transmembrane portion of the β subunit, while the benzodiazepines modulate the protein through interactions with transmembrane amino acids between the α and γ subunits near the intracellular side. NMDA receptors are activated by the agonist glutamate and co-agonist glycine only when voltage changes displace Mg^{2+} from the ion channel pore. Ketamine also acts primarily by a pore-blocking mechanism.[19] Reprinted from Pharmacology and Physiology for Anesthesia, edited by Hemmings HC, Egan TD, Intravenous Anesthetics, Garcia P, Whalin MK, Sebel PS, Pages 137-158, Copyright 2013, with permission from Elsevier.

The excitatory signaling in the adult mammalian CNS is primarily dependent upon the neurotransmitter glutamate acting on *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors.[46,61] The glutamate NMDA receptor is a heteromeric, integral membrane protein subclass of the excitatory L-

glutamate family of neurotransmitter receptors, acting via the receptor-gated cation channel, which is permeable to Ca^{2+} and some monovalent cations[48,61] such as sodium, to depolarize neuronal membranes and activate signaling pathways.[21] The name "NMDA receptor" is attributed to the fact that NMDA stimulates these receptors under experimental conditions; however NMDA is not endogenous, it is a synthetic chemical used to probe the activity of this class of receptors.[62] Anesthetic effects are achieved by the blockade of NMDA receptors[19] when agents such as ketamine HCl,[63] phencyclidine (PCP),[11,64] dextromethorphan,[65] and nitrous oxide[66] act to antagonize the binding of glutamate.[11,63-66]

GABA Receptor Agonists: Propofol

The $GABA_A$ receptor agonist propofol is a short-acting IV anesthetic,[15,23,30,33-43] advantageous for its rapid action with short, predictable duration, in addition to rapid, antiemetic, clear-headed recovery.[15,23,30,33-43] The anesthetic effects of propofol are attributed to its high lipophilicity[43,67] allowing rapid penetration of the blood-brain barrier and distribution to the central nervous system.[38,67] The lipophilic nature of propofol in addition to the steric effects it exerts act to suppress excitatory synaptic transmission and synaptic plasticity, and facilitate long-term depression by increasing Cl^- conductance at the $GABA_A$ receptor.[43,46,49] Propofol is a non-chiral isopropylphenol[68-70] chemically distinct from all other IV induction agents.[70] Propofol has a pH of around 7.0, a pKa of 11, is stable at room temperature, and is not sensitive to light,[26,30] and due to high lipid solubility with only slight water solubility, propofol (Diprivan) is formulated as 1% w/v of oil/water emulsion of soybean oil, glycerol and purified egg phosphatide.[21,43] Propofol induces narcosis and suppression of withdrawal reflexes in a dose-dependent manner,[71] and in addition to antiemetic properties, propofol has antioxidant, immunomodulatory, neuroprotective, and analgesic effects,[43] and it is the only induction agent, with the exception of ketamine, with good analgesic properties in addition to its anesthetic action.[28]

Propofol may have other sites of action in addition to the $GABA_A$ receptor; the glycine receptor has been suggested to contribute to analgesia and anesthesia,[60] and some of the analgesic effects of propofol may be modulated through opioid receptors and the release of β-endorphin; serum levels of β-endorphin have been found to be increased during peak propofol action.[72] The cardiovascular and respiratory effects of propofol are similar to those produced by other injectable anesthetics such as barbiturates,[35] including brief periods of apnea, and reduction of arterial blood pressure and cardiac output.[19,33,40] Some adverse effects of propofol include pain on injection[30,73] (often mitigated with lidocaine), apnea, hypotension, and occasional excitatory effects such as myoclonus.[30]

Propofol does not accumulate significantly after repeat boluses, making it is especially suitable for long-term infusions during surgery, and for long-term sedation in the ICU.[19,21,26,30,73] Propofol is widely used as an anesthetic for gastrointestinal endoscopy and minor surgical procedures, as well as in obstetric and gynecologic practices for assisted reproduction procedures.[37] The most common obstetric procedures during which propofol is used are Caesarean sections[36,41] and transvaginal oocyte retrieval during in vitro

fertilization (IVF).[28,34,37] When used for IVF, propofol allows relatively painless puncture on an out-patient basis,[37] making it one of the most common anesthetics used in ultrasound-guided transvaginal oocyte retrieval.[15,34,37,39] Propofol is also a popular agent to reduce intracranial pressure (ICP)[73] and for neuroanesthesia[19] for its ability to decrease the cerebral metabolic oxygen requirement, allowing cerebral vasoconstriction.[19,73]

The anesthesia induction dose of propofol for human adults ranges from between 1.5-2.5mg/kg[19,26,30,43,67,74] with maintenance provided by continuous infusion of 4-12mg/kg/h or intermittent repeat bolus injections of 20-50mg,[43,67] and for mice, dosages range from 50-200mg/kg (IP) (50-200μg/mL).[75] In humans, the propofol distribution half-life for a single bolus or an infusion has been shown to be approximately 2 to 4 minutes, with total clearance between 77 and 156 L/h.[43,67] Metabolism of propofol is rapid[30,39,40,43,67] and primarily hepatic[30,40,43,67,76] and 88% of propofol metabolites have been recovered in the urine, with less than 0.3% excreted as unchanged propofol.[77] The major metabolites are inactive glucuronide metabolites,[43] the glucuronic acid propofol conjugate and the glucuronic acid and sulphate conjugates of its hydroxylated derivative, 2,6-diisopropyl-1,4-quinol.[30,77]

GABA Receptor Agonists: Barbiturates

Barbiturates are general central nervous system depressants used clinically for their anesthetic, sedative-hypnotic and anticonvulsant effects.[50,78,79] Barbiturates are substituted derivatives of barbituric acid, a condensation product of urea and malonic acid.[80] Barbiturates are neuromodulators which potentiate presynaptic inhibition by acting as agonists at the $GABA_A$ receptor[47,50,58,79,81] Barbiturates such as pentobarbital also act to enhance postsynaptic inhibitory responses to GABA by enhancing sodium (Na^+) independent binding of GABA to synaptic membranes at clinically relevant concentrations (100-1000μM).[78] Some common barbiturates include amobarbital, secobarbital,[24] methohexital , thiopental, phenobarbital (Luminal) and pentobarbital (Nembutal).[24,56,82] Although it has been largely displaced by benzodiazepines (BZDs), phenobarbital is still occasionally used for certain types of epilepsy and emergency convulsion treatment.[24,59] The more potent pentobarbital[56,58,71] was once a widely used general anesthetic, however the development of other anesthetic agents such as etomidate and propofol[24] in response to some of the negative anesthetic properties of barbiturates such as toxic effects including tissue irritancy or high mortality[83] has led to the reduction of the anesthetic use of barbiturates.[15,23,30,33-43] Pentobarbital anesthesia remains prevalent in some areas however,[82] and additional applications include treatment of insomnia as a sedative-hypnotic, emergency anticonvulsant treatment,[24,82,84,85] and treatment of intracranial pressure in patients with head injury, encephalitis, cerebral ischemia,[24,73,86] for sedation and treatment of ICP and epilepsy in pediatric intensive care,[73] and has been frequently used in pediatrics alone or combined with other agents for sedation during MRI procedures.[87] Pentobarbital also continues to be a common agent used for numerous applications in veterinary medicine and for laboratory animals[68,82,85,88]

The oxybarbiturate pentobarbital[26,68] is formulated the by the alkylation of α-ethylmalonic ester with 2-bromopentane.[89,90] The pentobarbital Nembutal solution is formulated as a sterile 6.5%[82] racemic mixture of stereoisomers,[82,89,90] containing propylene glycol for minor preservative action and to increase miscibility with water,[82,91] and like most barbiturates, it is formulated as a sodium salt to increase solubility and stability in solution.[19,21,82,92] Once reaching the plasma, barbiturates become lipophilic, leading to rapid profound anesthesia after bolus injection.[19] The recommended anesthetic dosage for pentobarbital is between 100-200mg (2-4mg/kg) for induction in humans.[24,73] For anesthesia in rodents, doses range from 30mg/kg (IV) for the rabbit[93] and 50mg/kg (50μg/mL) for mice (IP),[94] to the wide range of the rat, from 60mg/kg[95] to as much as 500mg/kg.[96] Pentobarbital is metabolized by liver microsomal P450 primarily by hydroxylation, resulting in four 5-ethyl-5-(3'-hydroxy-1'methylbutyl)-barbituric acid metabolites.[97]

NMDA Receptor Antagonists: Ketamine

D, 1-2-(o-Chlorophenyl)-2-(methylamino) cyclohexanone hydrochloride (ketamine HCl)[63] is a non-competitive antagonist of glutamate NMDA receptors.[21,28,29,68,98-100] Ketamine is an arylcyclohexylamine[30,101] PCP derivative, and a general parenteral anesthetic agent with unique pharmacological properties.[11,64] The ketamine molecule is chiral and is prepared as a racemic mixture of S (+) and R (-) enantiomers.[19,27,29,73,102] Ketamine HCl solution is freely water-soluble, slightly acidic (pH 3.5-5.5), with a pK_a of 7.5,[26,29,30] and benzethonium chloride is added as a preservative.[26] The rapid anesthetic action of ketamine HCl is attributed to its high lipid solubility and relatively low molecular weight (274.19).[29,31]

Ketamine is considered to be safe, nontoxic, and potent, with short duration of action.[7,100] Ketamine is widely used for anesthesia in pediatric patients,[29,103] and has been previously used in obstetrics for its rapid induction of profound anesthesia.[31,64] Currently, ketamine anesthesia is also common for patients suffering hemodynamic compromise due to the positive hemodynamic effects it exerts,[23,29] making its use is prevalent in military field hospitals,[27,29] and for emergency Caesarean sections with heavy blood loss.[104] The positive hemodynamic pharmacokinetics also make ketamine anesthesia useful for cardiovascular compromised patients with acute hypovolemia, hypotension, cardiomyopathy, pericarditis, congenital heart disease, bronchospastic disease, cardiac tamponade,[21] acute hemorrhage,[29] or acute asthma.[21,73] Ketamine has relatively low protein binding[19,82] much less than that of barbiturates or propofol, making it useful for hypoproteinaemic patients.[82] In addition, ketamine remains a common anesthetic in developing nations for its low cost,[19,27,29] and for veterinary surgery,[29,101] as well as being frequently used for sedation during regional techniques,[33] repeated dosage for analgesia in burn victims,[27,33] postoperative pain relief in patients with chronic symptoms,[33] and in pediatric patients.[19,26,27]

A unique attribute of ketamine is that it is one of the few anesthetics capable producing analgesia,[73] making its selection more desirable for certain applications than propofol or barbiturates.[26] The versatility of ketamine administrative routes (IV, IM, oral, rectal) also make its use advantageous,[21,26] especially in

pediatrics,[26] and it remains the only induction agent which can be administered intramuscularly (IM) as well as intravenously (IV).[23] CNS depression is the predominant effect produced by ketamine, and a broad range of doses can be injected to induce anesthesia without convulsions or danger of death from respiratory arrest,[63] and there is a low incidence of vomiting in the recovery phase.[31] Ketamine has a vasopressive effect on peripheral circulation[31] and has a short-lasting effect on the cardiovascular system causing tachycardia and an increase in arterial blood pressure.[12,31,63,64]

The main disadvantage of ketamine is a strong tendency to undesirable physiologic reactions that typically occur after awakening from anesthesia (emergence reactions).[28] These reactions are characterized as psychotomimetic side effects[31,32] and include vivid peculiar dreams, hallucinations, and delirium during recovery.[28, 31] Emergence reactions are less intense in children,[29] but the psychotomimetic effects in adults have led to the decreased use of ketamine as a lone agent.[23] Adverse reactions may be reduced by preoperative discussion with patients, but ketamine may also be used in combination with other drugs (such as BZDs)[26] for balanced anesthesia to significantly reduce hallucinations and other side effects.[23]

The recommended anesthetic dosage and for adult humans dosage ranges from between 0.5-2.0mg/kg (IV) and 4-10mg/kg (IM)[20,29] (maintenance with 10-30μg/kg IV infusion); and for sedation and analgesia, from 0.2-0.75mg/kg (IV) and 2-4mg/kg (IM) (with 5-20μg/kg IV infusion for maintenance).[29] For mice, the recommended anesthetic dosage is 50-100mg/kg (50-100μg/mL) administered via intraperitoneal (IP) injection.[105] Clearance of ketamine is rapid, resulting in a relatively short elimination half-life due to both the high hepatic extraction ratio and limited protein binding.[30] Ketamine is quickly metabolized by cytochrome P450 enzymes[19,61,64] by demethylation and hydroxylation of the cyclohexanone ring, with metabolites excreted in the urine.[29] The main metabolite of ketamine, norketamine, is one-third as potent as ketamine[19] and may contribute to the analgesic effects,[61] but also may be involved with the undesirable side effects.[30]

Choice Of Anesthetic

Injectable anesthetic agents may be used either to induce anesthesia prior to maintenance with a volatile inhalant, or as the sole anesthetic agent.[82] The most common inhalation agent is sevoflurane, however inhalation induction in is rarely used for anesthesia of adults.[20] Volatile anesthetic agents exhibit less specificity than IV agents,[19] but an ideal injectable anesthetic is yet to be developed.[82] For human anesthesia, use of a single agent is predominately achieved by IV injection, followed by infusion to extend anesthesia duration.[82] This anesthetic technique is known as total intravenous anesthesia (TIVA), and its development is largely attributed to the popularity of propofol as an efficacious monoanesthetic agent.[23] *TIVA provides good general anesthesia and eliminates the need for volatile agents,*[20,23] *however it is* necessary that drugs used for TIVA possess short half-lives to prevent accumulation.[20]

Rapid induction of anesthesia is the main advantage of IV agents, but all of the desired properties of an anesthetic may be unachievable with a single IV agent.[26] Some of the properties of a single agent may be

contradictory, such as the high lipid solubility of a rapid acting drug reducing its water solubility.[26] Some of these drawbacks may be addressed by presenting the agent as a salt to improve solubility such as in ketamine hydrochloride and sodium pentobarbital,[82,92] Solubility issues may also be alleviated by addition of other compounds, such as in the case of sodium pentobarbital, which contains propylene glycol to make it miscible with water,[82,91] or the lipid emulsion used to formulate propofol.[19] The addition of compounds to the anesthetic formulation can sometimes have important clinical implications, such as in the case of propofol, in which the lipid formulation can promote rapid microbial growth.[19] Other indications of anesthetic agents such as the positive antiemetic effects of propofol[19] and the analgesic effects[73] and versatility of ketamine,[21,26] or negative effects such as cardiovascular depression by propofol causing hypotension in some patients,[19] emergence reactions of ketamine,[28] or potential toxic effects of pentobarbital[83] influence the selection of an agent for a specific procedure. The availability of an agent is also an important factor influencing selection; the recent shortage of drugs such as propofol and thiopental has made the choice of an induction agent be more dictated by availability rather than by pharmacology.[21]

When available, the most ideal agents for TIVA include the alfentanil, as an analgesic and propofol as an anesthetic.[20] TIVA with propofol has advantages over anesthetic maintenance with volatile agents in some patient populations.[19] The rapid effects and antiemetic qualities of propofol make it a frequent choice for sedation in monitored anesthesia care, as well as for induction and maintenance of general anesthesia,[19] and it is especially suitable for long-term infusions during surgery and on the ICU for long-term sedation since it does not significantly accumulate.[26]

Many synthetic agents used for anesthesia are chiral drugs, and nearly all of these agents are distributed as racemic mixtures rather than in pure enantiomeric formulations.[102] One enantiomer is often be responsible for therapeutic action, and the other may offer different properties, be pharmacologically inert, or even have undesirable effects.[102] Ketamine and pentobarbital are produced as racemic mixtures, and most clinical preparations continue to be a racemic mix of the R and S enantiomers.[19,73] Enantiomers of ketamine have been found to retain desirable and lack undesirable properties of the racemic mixture,[27] and trials have been conducted with the (S+) enantiomer, which is more potent[106] and has been shown to limit potential adverse effects such as emergence reactions.[73,106] The The S(-)-enantiomorph of pentobarbital is more potent than the corresponding R (+)-isomer,[58,97] and future use of pentobarbital may be expanded by application of one stereoisomer if clinical experimentation shows promise. The disadvantage to use of single enantiomers is the availability of these isomers, which are expensive and scarce, which may also restrict identification of molecular targets of action.[107]

Preoperative Assessment, Pregnancy Testing, And Abuse

In addition to being one of the first physicians to adopt the practice of anesthesia, John Snow also introduced the importance of pre-anesthesia physical examination.[25] Between 0.5-2% of pregnant women are exposed to general anesthesia during non-obstetric surgery in the United States each year,[2-4] and this

percentage does not include patients who are not yet aware of their pregnancy at the time of surgery.[4] Basic standards exist for pre-anesthesia evaluations which apply to patients undergoing all techniques of anesthesia established by The American Society of Anesthesiologists.[25] These guidelines are periodically revised[25] to standardize the preoperative assessment and reduce the risk of critical incidents.[20]

The preoperative assessment is as important as the anesthetic procedure itself.[108] Preoperative assessment is the part of patient management before induction, and it establishes the extent of any co-morbidity, in addition to current medication, and potential complications which may arise in response to anxiety states, allergies, difficult airways or venous access,[20,108] diseases, or pregnancy.[108] Careful history evaluation and physical examination constitute routine preoperative assessment,[25,108] followed by further tests only if the indicated appropriate by patient medical history.[25] Routine tests may have shortcomings such as failure to uncover pathologic conditions, or inadequate follow-up and false positives may lead to patient distress and provoke further invasive testing.[25] One such test is the hCG pregnancy test, the most widely used immunoassay,[109] which is reliable but only necessary if indicated by the patient medical history.[25] Women of child-bearing age are asked about their last menstrual period and only if their pregnancy status is uncertain, pregnancy testing is performed.[4] The anesthesiologist must consider if the pregnancy would be affected by the anesthetic agents or if the pregnancy is desired or unintended, such as in the case of detection of pregnancy in teenager wishing to hide her condition from her parents.[25] If pregnancy is diagnosed, concerns are raised whether surgery or anesthesia may endanger the developing embryo or fetus by increasing the risk of congenital abnormalities or spontaneous abortion.[4]

The accurate establishment of the pregnancy duration and timing of conception is essential for optimum care.[110] First trimester anesthesia exposure has been found to increase the risk of spontaneous abortion and lower birth weight.[4] This is the period of maximum sensitivity to teratogenicity, during which organogenesis occurs.[4] To minimize potential miscarriage or congenital defects, elective surgery during this time should be deferred,[4] however surgery may sometimes be necessary in spite of the pregnancy.[3,5] Pregnancy may also be endangered by other factors in addition to surgery. 85% of prescriptions of potentially teratogenic medications to women of childbearing age are by outpatient physicians such as family/general practitioners, internists, psychiatrists, and dermatologists.[1]

Establishment of pregnancy relies upon several factors. Historically, pregnancy has been dated by using the first day of the last menstrual period (LMP), which is usually the only data available for very early pregnancy, but the LMP method is frequently inaccurate.[110] During early pregnancy, the rise in hCG concentration is uniform, so the measurement of hCG levels provides the most accurate early estimation of gestational age.[110] Home pregnancy testing using the hCG immunoassay offers the advantages of privacy and early detection necessitating a clinical visit.[111] More than 20 brands of home pregnancy tests (HPTs) are commercially available, and home pregnancy testing continues to be a dominant method for early detection despite about problems with interpretation or results and false pregnancy detection.[111] In a study of over-the-counter HPTs, only 1 of 18 HPTs tested was found to have the sensitivity needed to detect 95%

of pregnancies at the time of missed menses, and 2 gave false positive or invalid results.[111] Women with intended conceptions are more likely to recognize early signs of pregnancy than those with unintended pregnancies,[112,113] however the sensitivity limitations of HPTs causing false-negative tests can mislead women and result in avoidance of positive pregnancy behaviors.[109] The behavior of a woman during pregnancy may be influenced by their attitude toward the pregnancy.[112] This can affect the health of their infant by either compelling a woman to seek early prenatal care, or to avoid detrimental behaviors such as smoking,[112] use of fetotoxic drugs, or consuming alcohol.[109]

Nearly 90% of drug-abusing women are of childbearing age; consequently it is not unusual to encounter pregnant women who abuse illicit drugs.[114] Drug use may be explained by addiction or an individual's desire for novel experiences or changes in mood.[115] The diverse clinical manifestations of drug abuse in combination with physiologic changes of pregnancy or of a coexisting pregnancy-related disease may lead to significant complications.[114] The most extensively studied agent of abuse, alcohol, is well documented to have profound deleterious effects on embryonic and fetal development.[116-118] Heavy use of alcohol can also alter the effects of other drugs and may even increase euphorigenic effects.[115,116]

Drug abuse is not limited to illicit drugs with no recognized medical use; it also encompasses misuse and intentional abuse of pharmaceuticals and over-the-counter medicines. Legal pharmaceuticals may be illicitly obtained for misuse,[119] and even if a drug is obtained legally from a physician it may become subject to abuse.[119,120] While state and Federal law classifies (Schedules) and restricts access to controlled substances according to their medical use and potential for abuse, many of these substances continue to be abused.[121] In 2005, the National Survey on Drug Use and Health found 6.4 million people to have used prescription pharmaceuticals for nonmedical reasons during the past month, with use of pain relievers by 4.7 million, tranquilizers by 1.8 million, and stimulants by 1.1 million people,[122] and women have previously been found to have a higher rate of sedative and tranquilizer abuse.[123] Those granted access to controlled substances include chemical distributors, pharmacies, hospitals and clinics, physicians, veterinarians, and researchers;[121] however professionals that prescribe or work around restricted pharmaceuticals are at risk of abusing these readily available medications.[122] Professionals working in anesthesia,[119,124,125] emergency medicine, and psychiatry have the highest rates of pharmaceutical drug abuse,[119] and physicians in general also have a higher rate of psychoactive substance abuse than the general population.[124]

The increased abuse of substances by people working in medical and veterinary occupations may first attributed to access to controlled substances.[122,124-126] Many of these professionals administer these potent drugs and directly observe their effects on a daily basis, and often the routine of the process can make one desensitized to the importance and danger of controlled substances.[125] In addition, it takes a relatively short period of time to learn the technical skill to administer many of these substances, and often the application of these drugs is performed with minimal supervision.[125] This can cause some individuals without prior risk factors or history of abuse to develop a curiosity about these drugs, a sentiment which

may rapidly progress to experimentation and addiction.[125] In addition to anesthetics, some drugs commonly abused by medical professionals include opiates, amphetamines, barbiturates, and benzodiazepines.[119]

Even if some drugs such as veterinary medications are not approved for human use, they may be abused by individuals with easy access, including veterinarians or veterinarian assistants,[126-128] horse trainers, or farmers.[127] Like many physician and dental offices, veterinary offices maintain pharmacies to fill out-patient prescriptions and carry an inventory of drugs for on-site procedures.[129] Veterinary offices differ from most clinical medical offices, though, since they also serve as surgical centers, performing invasive procedures requiring large quantities of pharmaceuticals used for both human and veterinary medicine as well as drugs specific for animal use.[128,129] Veterinary offices are also unique in that they also perform euthanasia procedures requiring highly potent medications.[128,129]

Although the abuse of controlled substances may be higher in individuals working in occupations where these drugs are more readily accessible, many of these medications may be obtained by the general public by alternative means. Some individuals with access to controlled substances contribute to abuse through the illegal sale of pharmaceuticals.[122] In a recent survey of over 4,000 respondents, individuals were asked the source from which they illegally acquired their drug of choice. The three most frequently reported sources for illicitly acquiring a pharmaceutical drug were drug dealers, friends or relatives, and physician prescriptions.[130] Additional sources for illegally obtaining pharmaceuticals include theft,[130, 120] forged prescriptions, and the Internet.[130] While the Internet is currently a relatively minor source for the illicit purchase of prescription pharmaceuticals, many of these drugs are available on the Internet.[130] Most websites provide adequate control of the purchase of pharmaceuticals but the legality of some online sales is questionable and some websites are completely illegal.[131] Disadvantages of using the internet to acquire pharmaceuticals include fear of possible detection by authorities, scams, and price considerations (prescription drugs are less expensive on the street).[130]

Pharmaceuticals may also be illegally obtained in other countries where the restriction of certain agents is much less stringent than in the United States. An example of such an agent is pentobarbital, currently only used in the United States as a veterinary anesthetic[68,82,85,88, 128] and as a sedative/hypnotic in human medicine,[115] but its anesthetic use for humans continues in many countries.[82] In addition being subject to abuse, pentobarbital use for assisted suicide is advocated by the Exit International Association.[128] Exit's mission is to aide individuals experiencing chronic painful disease or severe physical degradation.[128] Information intended to assist individuals wishing to commit suicide is available in "The Peaceful Pill Handbook," written by the founder of the Exit Association, Philip Nitschke. In addition to containing extensive instructions for self-euthanasia, this publication also describes how to illegally obtain substances such as pentobarbital.[132] Nitschke cites Mexican pharmacies to be the preferred source to easily obtain pentobarbital and devotes an entire chapter detailing how to acquire and transport the drug back to the

US.[132] This information can be accessed in minutes following a simple web search by any individual lacking "street connections" with a desire to obtain pentobarbital.

Some of the side effects of anesthetics which have limited their use in the medical field can make them desirable to abusers.[7] Anesthetic use of pentobarbital is prevalent in veterinary medicine[121,128] but is limited in humans;[24,73,82,84-87,115] however it has a high potential for abuse[115,121,122,133] in addition to possessing strong addictive properties.[119,121,133] Common street names pentobarbital may be identified by include Nembutal, Nembies, yellow jackets, or yellows.[119] Another anesthetic used for both veterinary and clinical anesthesia, ketamine, is also highly addictive[119,121] and may possess an even higher potential for abuse.[7,11,29,101] Ketamine is considered to have unique euphoric hallucinogenic properties[7,119,134] that enable the abuser to experience effects similar to PCP, cocaine, or lysergic acid diethylamide (LSD) with varying doses; and it is frequently used in combination with other drugs and as an ingredient in MDMA (ecstasy).[7] Alternative names ketamine may be identified by include Special K,[7,11,29,101,116] Ket,[7,11,29,101] K, Vitamin K,[29,101] or cat.[119]

Propofol is currently a preferred general anethestic in human medicine[19,20,26] as well as being prevalent in veterinary medicine.[76,82] Recreational use of propofol has become an issue due to the mild euphoria and hallucinations it induces.[135] The abuse potential for the anesthetic propofol has not been completely defined, but numerous case reports exist in the literature.[70] Abuse of propofol is more prevalent among anesthesiologists due to its widespread use in clinical practice, and the short duration of action of propofol allows easy concealment of its abuse.[70] Recreational use of propofol is also significant among celebrities or patients who have become addicted following numerous elective surgeries, and a high incidence of death has been documented due to propofol misuse and abuse.[135]

Animal Use/Model

In humans, the teratogenic risk of drugs is difficult to assess,[5] and the teratogenic risk in human pregnancy is undetermined for over 90% of drug treatments.[136] Although drugs must undergo preclinical animal testing, these studies are not always predictive of effects in human pregnancy, and most clinical trials required for by the United States Food and Drug Administration (FDA) approval focus on immediate adverse effects and exclude pregnant women.[136] While consideration of potential harmful effects to a pregnancy is ubiquitous, prescriptions of potentially teratogenic medications to women of childbearing age by outpatient physicians is frequent.[1] Pregnant woman may also be exposed potentially harmful drugs during surgery spite of her pregnancy.[3,5,136] Women such as those with intended conceptions are more likely to recognize early signs of pregnancy than those with unintended pregnancies,[112,113] and their attitude toward the pregnancy can influence the health of the embryo by avoiding detrimental behaviors such as use of potentially fetotoxic drugs or postponing elective surgical procedures.[109] Negligence of early pregnancy due to the sensitivity limitations of HPTs[109] or disregard of potential harmful effects due to attitude or addiction may cause some women to continue to abuse illicit drugs.[114] Though teratogenic studies may not

find an anesthetic to pose significant risk, exposure conditions of these studies are not comparable to binge exposure conditions.[137] In addition, procedures of assisted reproduction such as in vitro fertilization (IVF) require the use of anesthetic drugs with potential toxic effects when exposed to the oocyte or early embryo.[10,138]

Consideration of the potential teratogenic effects of drugs used in veterinary medicine and for laboratory animals is also important. In laboratory animals and veterinary anesthesia, injectable agents are preferred for a variety of reasons including the minimization of equipment, ease of administration, and fewer safety concerns than those posed by use of inhalants.[139] Common injectable anesthetics used for laboratory animals and in veterinary medicine include pentobarbital,[68,88] ketamine, and propofol.[76,82] The decades of use of pentobarbital, in addition to its generalized availability, low cost, relatively rapid onset of anesthesia, and ease of injection (IP or IV) to a variety of animals contributes to its continued widespread use in laboratory animals and in veterinary medicine,[88] and its use is especially prevalent for rodent anesthesia.[68,88] The use of ketamine is widespread throughout veterinary anesthesia, and propofol is commonly used for feline and canine anesthesia.[76,82]

Human IVF programs require continuous quality controls for the maintenance and improvement of reagents and methods.[140-143] Due to the complex nature of IVF, an animal model which allows for the testing of drugs, routine preparations of reagents, skills of technicians, and innovative methods is required.[140,141,143-146] The most common animal model for this purpose is the mouse embryo growth and development bioassay, in which the development of in vitro and in vivo fertilized 2-cell mouse embryos to the hatching blastocyst stage is tested.[144,145] While lower order species such as the zebrafish,[147,148] frog (Xenopus),[149] and sea urchin are occasionally utilized for studying teratogenesis, fertilization, and embryo development, the mouse is advantageous for mammalian reproductive studies.[138] The mouse is a superior animal model for mammalian embryonic study since the mouse embryo divides slowly after fertilization,[138] contributing to the collection of abundant knowledge of mouse genetics.[138,150] The mouse system is also valuable for the fundamental similarities between mouse and human preimplantation development and implantation processes.[150] Collection of embryos at the 2-cell stage is performed because embryos at this stage are much more resilient in culture than those collected at the zygotic pronucleate stage.[151] Like human embryos, mouse embryos are able to develop in simple defined media in vitro,[150] allowing the mouse embryo to be studied extensively as a model for mammalian embryogenesis.[152] For this reason, the mouse embryo growth and development bioassay is preferentially used for the screening of drugs, and is considered to be by the clinical reproductive industry and the FDA, the preferred assay for quality assurance/quality control (QA/QC) determination and validation of gamete and embryo research,[153] as well as to improve pregnancy rates.[141,154] Although the FDA has not prescribed a standard procedure for test performance and design, it has described the mouse embryo assay to be the most appropriate indicator of potential toxicity of materials used for assisted reproduction.[143]

All aspects of laboratory procedures require meticulous attention to increase the chance for a patient to successfully obtain pregnancy.[145] IVF technologists must be trained and experienced in proper tissue culture techniques for handling mammalian gametes and embryos before working with human tissues.[145] The mouse embryo culture system is utilized to compare the ability of different media and supplements to support mammalian preimplantation embryogenesis, or other components used in IVF programs such as culture dishes, collection devices, pipettes, surgical instruments, and surgical gloves;[141,142,144] or to test the effectiveness of instrument sterilization procedures.[142,144] Media is required to support at least 75% morula or blastocyst Development of 2-cell mouse embryos following a 72h culture period to be approved for use in human IVF,[141,144,145] with no more than 15% degenerating or fragmenting.[144] This does not offer direct proof that human fertilization or embryogenesis would be impaired, and is not able to predict success for human IVF,[141] rather the mouse bioassay assists in screening and identifying potentially failure-causing problems before the media are used with human gametes or embryos.[144]

The in vitro culture mouse embryo assay may also aid assessment of safety of drugs[155] and can be used to screen potential toxic effects of anesthetics used in egg retrieval for IVF,[10, 138,143] or those of pharmacologic agents that may come in contact with the egg or early embryo.[10,138,146] Prediction of the safety risk of drugs is challenging due to the limited and restricted availability of human cells for toxicity evaluation.[156] The preimplantation mouse embryo in vitro development bioassay helps to elucidate the effect of a pharmacologic agent on cellular differentiation;[138,146] and the zygotic and early embryonic stages provide indications of possible experimental induction of fetal and postnatal defects.[157] In addition to economic benefits of the in vitro mouse embryo assay,[155,158] it assists the expansion of the range of data obtained by toxicological assessments.[146,155] Numerous attempts to culture embryos continuously from the preimplantation to post-implantation developmental phases have been made,[149,158-162] but these tests have not been widely adopted for the study of mammalian embryogenesis.[148,155] These tests are relatively complex, require high technical skills, and are costly.[149]

Developmental toxicity testing is expensive, time-consuming,[147,148] and requires a large number of animals.[148,163] The necessity to improve testing methods has led to development of a number of alternatives, but the complexity of embryogenesis has made development of less animal-intensive alternatives challenging.[147,148,163] Aside from culture of non-mammalian and mammalian embryos to test for developmental toxicity,[138,146,148,149,162,163] alternatives include testing of sperm motility and viability,[145] mammalian micromass (MM) cultures[162] and embryonic stem cells.[147,148,156,162-164] The MM culture test is based on detection of the ability of a particular chemical to inhibit the formation of foci of differentiating cells within a background of undifferentiated mesenchymal cells.[162] MM culture allows detection of cell division, differentiation, adhesion, movement, and communication, and the MM culture represents a promising model for the study of teratogenesis.[162]

The embryonic stem cell test (EST) is one of the most extensively studied alternative in vitro methods for testing developmental toxicity.[148,164] In the EST, mouse blastocyst-derived pluripotent embryonic stem

cells to assess teratogenic risk.[162] The EST makes use of an established permanent cell line[147,162] and is designed as an alternative to animal testing.[156] Compared to other in vitro embryotoxicity tests, the advantage of EST is that it does not require animal use after cell line establishment,[147] and consequently no additional animals are sacrificed to obtain embryonic cells or tissues.[148,162] The disadvantage of the EST is it gives a reductionist representation of embryonic development and evaluates an effect on only a single endpoint.[147] Although it has been validated, the applicability and predictability of the EST model requires further study for successful implementation as an alternative toxicity testing method.[164] Due to the complexity of the reproductive cycle, the hundreds of signaling pathways involved,[163] and the multiple steps involved in embryonic development such as cell proliferation and differentiation, pattern formation, and organogenesis, it is impossible to develop an alternative test that can encompass the entire developmental process with a single comprehensive test.[147,148,149]

Preimplantation Embryo Development

Fertilization in mammals initiates a series of cleavage divisions which partitions the zygotic cytoplasmic mass into successively smaller cells.[165] The zygote is enclosed in an acellular glycoprotein envelope, the zona pellucida,[166,167] and cleavage division to the blastocyst stage occurs within the zona pellucida.[167] Early embryogenesis consists of two distinct phases separated by the event of uterine implantation,[155] which occurs during the final period of the cleavage phase, the blastocyst stage.[167,168] The cleavage phase of development leads to the separation of two tissue types, the inner cell mass (ICM or embryoblast), and the trophoblast (trophectoderm).[169] The ICM differentiates into two layers, the epiblast, from which cells will develop to form the body of the embryo, and the hypoblast, responsible for extra-embryonic tissues.[155] Additional extra-embryonic tissues responsible for providing support for uterine development are derived from the trophoblast.[155]

Within 24h of fertilization, male and female pronuclei replicate their DNA in the 1-cell zygote, their chromosomes congress at syngamy, followed by mitosis, cytokinesis, and formation of the 2-cell embryo.[170] Division of cells (blastomeres) is asynchronous, but initially each division is completed before the next round begins, giving rise to intervals of 2-, 4-, and 8-cell stages.[165] In mice, the first round of cleavage occurs 1 day after fertilization to produce 2-cell embryos, at which time researchers typically collect embryos.[171] Each blastomere is totipotent during the 2-cell stage.[172] In the 2-cell embryo, each blastomere has an oval shape with clearly defined polar bodies.[171] During the next two cleavages, each blastomere is easily distinguished and has a spherical shape,[173] and blastomeres are only loosely associated.[166] During divisions at the 8-cell stage, adhesion of blastomeres begins,[171] blastomeres change shape and spread over each other to maximize intercellular contact, and the embryo begins to compact to form a morula.[165] Adhesion occurs by the formation of adhesion complexes such as adherens, gap and tight junctions,[170] and allows physical attachment, cell-cell communication, and signaling for cellular differentiation.[171]

During the Ca^{2+}-mediated compaction[173] of the morula (16-cell embryo), outer and inner cells begin to develop,[169,171] causing a major change in embryonic physiology. This change is due to the formation of epithelium and initiation of a more somatic cell physiology by the embryonic cells.[151] Small fluid-filled cavities formed by active transport of water and ions begin to develop, and these cavities converge to form a space called the blastocoel.[169-171] The outer cells of the trophectoderm are responsible for this Na^+/K^+-ATPase dependent[170] internalization of fluid which generates the blastocoel,[165,169] to form the blastocyst and help prepare the embryo for implantation.[165] This blastulation event coincides with the completion of the fifth round of cleavage (at the 32-cell stage) in the mouse, approximately 4 days after fertilization.[165,170]

Formation and expansion of the blastocoel is crucial to the differentiation of the ICM and "hatching" of the embryo from the zona pellucida for further development to occur to prepare for uterine implantation.[152,174] Implantation has been found to occur when mouse blastocysts exceed approximately 140 cells,[168] usually within 36-48h of blastulation.[167] In vitro culture of mouse embryos is possible for nearly one-third of the 19 day gestation period, until the end of the cleavage stage at which time the blastocyst is prepared for implantation,[152,159,160] however without embryonic contact with a living uterus, further culture is extremely difficult,[151,155, 159,175] and continuous culture of mammalian embryos throughout pre- and post-implantation phases has not been widely adopted.[155]

Physiology Of Mouse Preimplantation Embryo Development

During the preimplantation period of mammalian development, the embryo undergoes significant changes in physiology, metabolism, and genetic control.[151] Within 24h of fertilization, DNA of the male and female pronuclei is replicated in the 1-cell zygote.[170] This is followed by syngamy, when chromosomes of the pronuclei congress, then mitosis and cytokinesis occurs to form the 2-cell embryo.[170] In the initial stages following fertilization, transcription from the embryonic genome is essentially absent, and embryogenesis is regulated by maternally inherited components.[176,177] Encoded by maternal-effect genes, these maternal proteins and mRNAs accumulate during oogenesis and regulate the activation of the embryonic genome and subsequent cleavage stages of embryogenesis.[170,177,178] This period of post-transcriptional maternal control extends from fertilization to the mid 2-cell stage in mice,[178,179] (4-cell stage in humans)[178] when the embryonic genome is activated.[151,176,180]

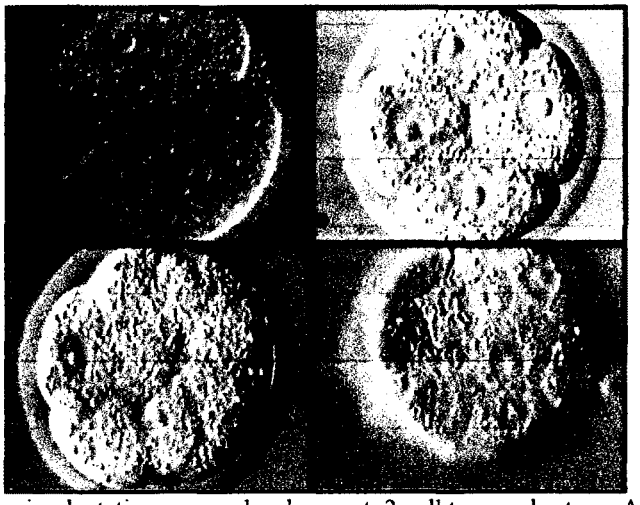

Fig. 2. Summary of the preimplantation mouse development, 2-cell to morula stage. After fertilization, the 1st round of cleavage yields the 2-cell stage (A). The 2nd round results in the 4-cell embryo (B), and the 3rd cleavage results in the 8-cell (C). During the 8-cell stage, cell–cell adhesion increases and each blastomere is not easily distinguished (beginning of compaction). During the next two rounds of cleavage (morula, 16- to 32-cell stage) (D), small cavities develop and fuse to form the blastocyst cavity. Photomicrographs taken using DIC Optics at 400x.

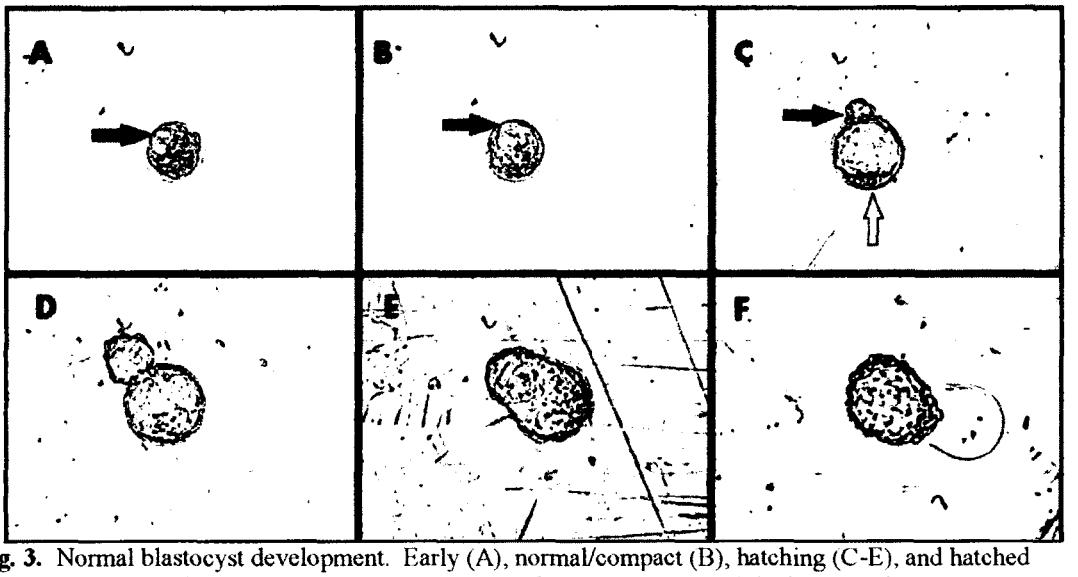

Fig. 3. Normal blastocyst development. Early (A), normal/compact (B), hatching (C-E), and hatched blastocyst (F). Cavities between blastomeres fuse to form a space termed the blastocoel (dark arrows in A and B). Contractions of the blastocyst within the zona pellucida contribute to its "hatching," embryo eruption from the glycoprotein coat (dark arrow in C). The blastocyst contains two cell types, the ICM (evident at tip of hollow arrow in C), and the trophectoderm, the surrounding outer layer of cells. Photomicrographs taken using DIC Optics at 200x.

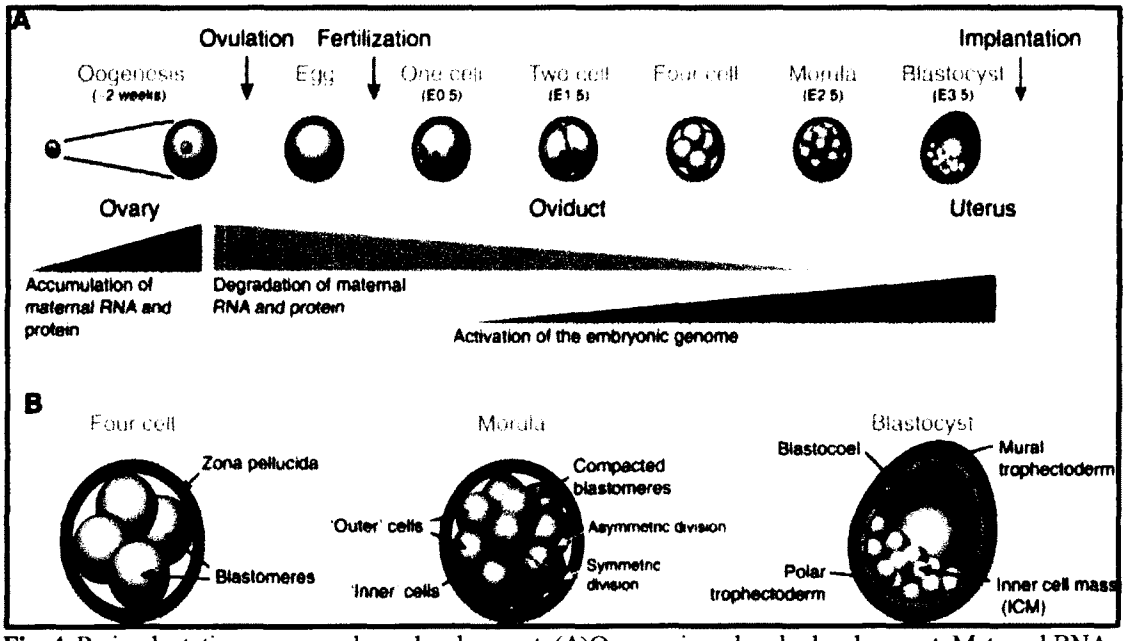

Fig. 4. Preimplantation mouse embryo development. (A)Oogenesis and early development. Maternal RNA and proteins accumulate within individual oocytes during a ~2 week growth phase, but many are degraded during meiotic maturation and ovulation. The zona pellucida surrounds growing oocytes and ovulated eggs (green) and is modified following fertilization (red) to prevent polyspermy and to protect the embryo as it passes through the oviduct. At embryonic day 0.5 (E0.5), 1-cell embryos can be recovered from the oviduct and, by the 2-cell stage (E1.5), there is robust embryonic genome activation. Following the third cell division, embryos undergo a $Ca2^{+}$-mediated compaction to form morulae (E2.5), and develop a fluid-filled blastocoel cavity from the 32-cell stage to become blastocysts (E3.5) that implant in the uterus wall (E4.5). (B) Late preimplantation development. At four cells, blastomeres with distinct cell boundaries are encased within the protective confines of the zona pellucida. Individual cells remain pluripotent. Following the 8-cell stage, the embryo compacts to form the morula, with increased cell contact that morphologically obscures cell boundaries. Symmetric cell divisions result in similar daughter cells. Asymmetric divisions provide topologically distinct outer cells, which are trophectoderm precursors (forming the placenta), and inner cells, which establish the inner cell mass (ICM) of the fetus. Blastocoel formation defines the blastocyst, and the eccentric localization of the ICM localizes the polar and mural trophectoderm, the latter of which initiates uterine implantation.[170] Reproduced with permission, *Development.* Li L, Zheng P, Dean J: Maternal control of early mouse development. Development 2010; 137: 859-870

The critical event that governs the transition from maternal to embryonic control of development, known as zygotic genome activation (ZGA),[181] is arguably the first event following fertilization necessary for further successful development to occur.[177] ZGA initiates the endogenous transcription of the embryonic genome during the late 1-cell stage[176,177] 1-2h following initiation of S phase in the 1-cell embryo.[182] Transcription steadily increases thereafter,[182] with the rate of transcription dramatically escalating during the 2-cell stage.[177] ZGA is involved in replacing maternal transcripts that are common to both the oocyte and embryo, as well as generating new transcripts that are unique to the developing embryo.[177] Transcriptional reprogramming during ZGA at the transition from the 2-cell to the 4-cell stage is also particularly important in preimplantation embryos.[183] Some of the gametic genes are regulated

during reprogramming by noncoding RNAs or CCCTC-binding factor-dependent insulators.[184] ZGA also requires chromatin remodeling, during which proteins restructure or move nucleosome transcription constraints, making the genome accessible to transcription factors such as heat shock factors (HSFs), and zinc-finger proteins such as basonuclin 1 and CCCTC binding factor.[170] HSFs encode heat shock proteins (HSPs), highly conserved cellular stress proteins.[178] HSPs are a critical component of a cellular defense mechanism in response to adverse environmental conditions such as physical or chemical insults; however under physiological conditions, HSPs act as molecular chaperones[178] which help to regulate cellular homeostasis.[185] Basonuclin is a transcription factor[170,186] abundant in germ cells, affecting RNA polymerase-mediated transcription, and CCCTC-binding factor is a DNA-binding protein ubiquitously expressed in mice.[170]

Differentiation, compaction, and blastocoel development are dependent upon the transcription of appropriate embryonic genes, and all of these processes require an increase in the level of total biosynthesis and energy demands of the embryo.[166] Metabolic control during preimplantation development may be either intrinsic to the embryo or extrinsic, mediated by the environment within the female tract.[187] Preimplantation embryos exhibit metabolic adaptation, in which their metabolism responds to changes in the external environment.[187] One of the characteristics of metabolic adaptation displayed by preimplantation mouse embryos is the metabolic shift from a dependence on the TCA cycle during the pre-compaction stages to a metabolism based on glycolysis between the late morula and late blastocyst stages.[179,188,189] Like the oocyte, the zygote possesses low metabolic activity, low levels of oxygen consumption, and low QO_2,[151] and prior to compaction, glucose is not utilized as the main energy source.[166] Instead, uptake of pyruvate and amino acids such as glutamine is necessary for ATP production, and this remains dominant until the 8-cell/morula stage, when glucose consumption increases dramatically.[179,190] Embryonic glucose uptake increases through blastocyst compaction, and by the blastocyst stage, glucose is utilized as the primary energy substrate.[166] This transition is largely attributed to the marked increase in ATP requirements necessary for blastocoel production, due to the active transport of ions mediated by the trophectoderm sodium pump (Na+, K+, ATPase).[191] While not utilized as the main energy source until compaction,[166,190] glucose uptake has been detected in earlier stages.[179] Several glucose transporter (GLUT) isoforms have been detected in early mouse embryos;[192,193] these GLUT isoforms exist to mediate a facilitated-diffusion glucose transport system.[194] The isoform present in nearly every tissue, GLUT1, is expressed throughout preimplantation development, and is thought to provide the embryo with its basal glucose requirements;[193] however additional GLUT isoforms including GLUT2, GLUT3, and GLUT8 are intrinsically expressed from the 8-cell stage to the blastocyst stage.[195]

Mitochondrial function is also critical to the initiation and progression of early development.[190] Mitochondria are responsible for providing the energy required to maintain cellular activity through oxidative phosphorylation and provision of intermediates for glycolysis; as well as affecting ion homeostasis, participating in amino acid metabolism, signal transduction, and apoptosis.[196] Mitochondria

also regulate the redox-balance;[197] however free radicals can damage mitochondrial membranes, causing new sources of harmful reactive oxygen species (ROS) to be generated.[198] Apoptosis is the physiological process of regulated cellular destruction,[199] and is responsible for the normal turnover of somatic cells,[200] as well as removal of excess, misplaced, damaged, or genetically abnormal cells in the embryo.[178] Unlike necrosis, apoptosis affects individual cells;[178] cells undergoing apoptosis fragment into membrane-bound apoptotic bodies, which are eventually phagocytized by surrounding cells or macrophages without inflammatory response.[69,178] Apoptosis is a necessary process for early embryo survival, and the first apoptosis in the preimplantation embryo may take place as early as during the activation of the embryonic genome.[178] Anti-apoptotic BCL2 and pro-apoptotic BAX proteins have been detected in zygotes and every subsequent stage of preimplantation development,[201] but the early preimplantation embryo is resistant to pro-apoptotic signals due to biochemical properties of the mitochondrion and nucleus.[202]

Anesthetic Effects On Development

Teratogenic effects may result from exposure to environmental factors such as radiation, dietary exposure such as alcohol, or pharmaceutical exposure to drugs.[1] Numerous studies document the potential detrimental effects to development following exposure to alcohol and anesthetics.[2,6,7-15,116,117] Most anesthetics are lipophilic with low molecular weight, increasing their ability for placental transfer.[76] Early embryonic development may be disrupted by exposure to exogenous agents, particularly anesthetics used for emergency surgery, obstetrics, or assisted reproduction procedures such as IVF. Ultrasonically-guided transvaginal oocyte retrieval for IVF is one such procedure during which xenobiotic agents may have effects on development. Oocyte retrieval can be accomplished with either heavy intravenous sedation or spinal anesthesia,[203] however since oocyte retrieval is a relatively short procedure (normally completed within 30 minutes), rapid and short-acting anesthetic drugs are preferred.[204] Some of the commonly used drugs for IVF, including midazolam,[138] alfentanil,[204] and propofol[10,37,39, 204,205] have been detected in the follicular fluid of patients, demonstrating their ability to come in contact with an oocyte or a preimplantation embryo in uterine fluids. In addition, as many as 2% of pregnant women require surgery not related to IVF,[3] and many of the common induction agents used during these surgeries have the capacity to come in contact with a developing embryo. During early pregnancy, the risks of anesthetic embryotoxicity and teratogenicity are much greater depending on the stage of embryogenesis and exposure duration.[76] Exposure to harmful agents may also occur as a result of substance abuse during pregnancy.[116] Therefore, it is necessary to investigate the profound effects which may be exerted upon fertilization, cleavage and proliferation, differentiation, implantation, and subsequent embryonic and fetal development in the presence of exogenous compounds.

Some drugs, such as the very short-acting opioid alfentanil,[204] or the benzodiazepine anesthetic midazolam, seem to offer ideal properties with little adverse effect on in vitro development.[138] During the oocyte retrieval procedure, the alfentanil concentrations in follicular fluid are about ten-fold smaller than

serum alfentanil concentrations at the same time points.[204] Although oocytes are exposed to alfentanil,[204] it has not been found to produce adverse effects on fertilization or embryo quality.[206] Midazolam provides conscious sedation for patients undergoing egg retrieval for IVF, and has also been found in patient follicular fluid.[138] In a 72h study where midazolam was cocultured with 2-cell mouse preimplantation embryos, no adverse effects were found on in vitro development of 2-cell to blastocyst stage embryos at concentrations approximating and exceeding those at which ova are exposed to during clinical anesthesia.[138]

Other anesthetics which may come in contact with the oocyte or embryo, such as the local anesthetic lidocaine, have also been found to adversely affect in vitro development of mouse embryos.[9] In studies of 72h mouse preimplantation embryo exposure effects, the percentage of 2-cell embryo cleaved and developed to more advanced stages was found to be significantly reduced by prolonged exposure to lidocaine.[8,9] Development to the blastocyst stage was inhibited by all lidocaine doses tested in vitro, and most affected embryos showed arrest, degeneration, and clastogenic damage.[8]

Even brief exposure of preimplantation embryos to anesthetics may be deleterious to subsequent embryo cleavage, and effects are highly dependent on the developmental stage during which exposure occurs.[2] Nitrous oxide has long been used as an inhalation anesthetic,[23] and like ketamine, acts as an NMDA receptor antagonist.[66] First trimester nitrous oxide exposure has demonstrated an increased incidence of skeletal defects and spontaneous abortions.[76] Nitrous oxide has also been shown to inhibit preimplantation mouse embryo development. Nitrous oxide was used to expose 2-cell, 4-cell, and morula stage embryos to determine effects on subsequent development after short durations of exposure.[2] All groups were exposed to 60% nitrous oxide/40% oxygen for 30 minutes, but 2-cell embryos treated within 4h of expected cleavage were the only group where effects were observed. At 3-4h and 0-1h prior to the anticipated onset of cleavage, exposure of 2-cell embryos to nitrous oxide resulted in blastocyst development in 27.7% and 4.7% of embryos, respectively, while 75%-77% of control embryos reached the blastocyst stage.[2] In addition, most affected embryos were halted at the 2-cell stage, with no further cellular division.[2] Similar effects were obtained with 80% nitrous oxide/20% oxygen, leading to the conclusion that even short-term nitrous oxide treatment of 2-cell mouse embryos significantly inhibited subsequent cell division.[2]

Isoflurane, another inhalation anesthetic, also has shown increased incidence of skeletal defects and spontaneous abortion following first trimester exposure.[76] Isoflurane was also found to adversely affect subsequent preimplantation development when 2-cell mouse embryos were exposed just before the onset of their first cleavage in vitro.[6] Development to the blastocyst stage was inhibited by 3% and 5% isoflurane when 2-cell embryos were exposed 3-4h or 0-1h before the onset of cleavage.[2] Most of the affected embryos were found to arrest at 3- to 4-cell stage, but the development to blastocyst of embryos exposed to isoflurane at the 4-cell or morula stage was unaffected.[6] Another study found the combination of isoflurane/nitrous oxide to affect fertilization and preimplantation development as well.[13] Patients

undergoing bilateral laparoscopic tubal sterilization were subjected to inhalant general anesthesia with isoflurane/nitrous oxide.[13] Sera was collected 1h after anesthetic induction, and its addition to the in vitro culture media was found to significantly reduce the amount of 2-cell mouse embryos developing to the blastocyst stage, as well as being associated with a significantly decreased fertilization rate of mature oocytes.[13]

Propofol has been shown to display similar pharmacokinetics to alfentanil when used as an anesthetic,[204] but the two act via different mechanisms, alfentanil is an opioid[204] and propofol is a $GABA_A$ receptor modulator.[207] While propofol is one of the most common IV anesthetics for general anesthesia[15,23, 30,33-43] and in ultrasound-guided transvaginal oocyte retrieval,[15,34,37,39] little is known about the effects of propofol on embryo development and pregnancy.[15] Conflicting reports of the impact of propofol on oocytes and early embryos leave questions about the effects of exposure to during early development. Although concentrations in the follicular fluid have been found to be approximately ten-fold smaller than serum concentrations,[2,204] propofol use has been found to have no detrimental effect on assisted reproduction outcome.[208] One study found no significant evidence to support a negative impact of propofol on oocytes, measured by cumulative embryo scores, probability of clinical pregnancy, and implantation rate following administration during IVF procedures.[209]

Propofol has been shown to negatively affect fertilization and subsequent cleavage, interfering with the ability of the oocyte to fuse with spermatozoa, without affecting post-fusion events such as sperm decondensation and cell cycle resumption.[15] Treatment of 1-cell embryos for 14h with propofol concentrations ranging from 0.01 to 10μg/mL was found to result in the inhibition of cleavage to the blastocyst stage, but 14h exposure of embryos at the 2-cell stage to even 10μg/mL did not impair subsequent development to blastocyst.[15] Embryos at the 2-cell stage are more robust in culture than the 1-cell zygote,[151] and these effects were attributed to cell cycle differences between the 1-cell and 2-cell embryos. The G1 phase is much longer in the zygote (4-8h) than in the 2-cell embryo (1-2h),[15,210] and since cells at the G1 phase of the cell cycle are particularly susceptible to environmental factors, a prolonged period of sensitivity may have contributed to the inhibition of differentiation in the 1-cell embryos.[15]

A second in vitro study of mouse oocytes found short-term exposure to propofol deleterious to fertilization in a dose-dependent manner,[37] even during brief exposure.[10] Mouse oocytes were exposed in vitro to propofol concentrations ranging from 50ng/mL to 5,000ng/mL for 30 minutes, then washed, inseminated, and assessed for ability to fertilize and subsequently develop to the blastocyst stage.[10] When fertilization did occur, propofol was found to significantly affect subsequent embryo cleavage and development to the blastocyst stage.[10] Propofol exposure was also found to result in parthenogenetic activation of 33% to 60% of oocytes.[10]

Exposure of early mouse embryos to propofol has been found to produce an adverse effect on embryonic development,[10,15] but later effects on the fetus have yet to be determined.[35] Propofol has the ability to cross the placenta,[67] making the study of fetal exposure an area of interest. During the postnatal

period, a high dosage of propofol (25mg/kg) was found to induce complex physiological changes in the developing rat brain, accompanied by age-dependent neurodegeneration in the cortex and thalamus at postnatal day 7,[211] but no evidence of teratological effects has been found in the rat or rabbit at lower doses ranging from 5-15mg/kg.[43]

When extrapolating the results of fetal and postnatal exposure from animal studies, it is important to consider the timing of development. Like the development of most tissues, the primary period of neurodevelopment differs between species.[212] In rodents such as rats, synaptogenesis occurs mostly during to the postnatal period, from 1 day before birth until approximately postnatal day 14 day; whereas in humans, this period occurs during the third trimester into the first several postnatal years.[212,213] Many environmental agents such as ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, benzodiazepines, halothane, isoflurane, propofol, and nitrous oxide, as well as pharmaceuticals used in obstetric and pediatrics medicine as sedatives, anti-convulsants, or anesthetics have the potential to induce neurodegeneration during development, and it is likely that development of other tissues may also be affected.[118,214]

Barbiturate degenerative effects on neurological development[84,85,116,215-218] and induction of congenital deformities are well documented,[216,219] but reports of effects on the development of the preimplantation embryo are sparse. The anesthetic thiopental has not been reported to produce negative effects to human development,[76] but sodium pentobarbital has demonstrated potential for embryotoxicity. In a study to evaluate the reproductive effects of sodium pentobarbital on the early embryo, female rabbits were injected intravenously at either 15 minutes or 6h after mating.[14] Unlike mice, rabbits are induced ovulators, releasing their ova approximately 12h post coitus.[220] At 17h or 24h post-coitus, oviducts were flushed and the developmental stage of the zygotes was observed and recorded, and blastocysts were also recovered from females on gestation day 6. A greater frequency of chromosomally abnormal blastocysts was found to develop in animals injected with pentobarbital than that found in controls.[14]

Ketamine is a widely used anesthetic in pediatrics,[103] and has been commonly used in obstetrics for Caesarean sections,[104] and ketamine abuse is prevalent,[116] making study of its effects on embryonic and fetal development essential. Although ketamine has not been reported to have negative effects on human fetuses,[76] it easily crosses the placenta and is rapidly distributed in the fetal tissues.[31,64] The rapid transplacental passage of ketamine may be attributed to its relatively low molecular weight, and ketamine concentration in the umbilical cord has been found to be higher than in the blood of the mother.[31] Ketamine has also been found to produce uterine artery vasoconstriction, a fall in uterine blood flow, and eventual fetal deterioration.[12] Administration of the drug has stimulating effects on the fetus causing rise in fetal arterial pressure and heart rate;[64] however, no evidence of fetal hypoxia, metabolic acidosis, or arrhythmia has been found following ketamine application.[12]

Ketamine and its metabolites have been found to contribute to developmental defects in rodents; however toxicity of ketamine in the preimplantation embryo is unreported. Observations of anatomical

effects of ketamine on development in CF-1 mice have shown daily 50mg/kg doses from gestation day 6-15 to produce insignificant skeletal and visceral defects, as well as no increased resorptions, dead fetuses, or decreased fetal weight.[7] Histological examination of the heart, liver, and kidney, however, has revealed focal nuclear hypochromatosis, interfibrillary edema, parenchymal cell degeneration, and proximal convoluted tubule degeneration.[7,11] The degenerative effects of ketamine have also been found to be dependent on the dose and duration of treatment, including effects in liver, kidney, and heart of rat pups exposed to daily doses of 25mg/kg, 50mg/kg, and 100mg/kg on days 1-15 and days 5-15 of gestation.[11]

Fig. 5. Degenerating 2-cell mouse embryo following 72h of culture with 1000μg/mL ketamine HCl. Irregular shape of blastomeres and cellular fragments smaller than normal-size blastomeres are indications of degeneration and apoptosis. Photomicrograph taken using DIC Optics at 200x.

Mechanisms Of Embryotoxicity

Drugs must act upon or cross cell membranes to produce their effects,[45] and one of the qualities of a rapid-acting IV anesthetic, high lipid solubility[24,26,45] produces transfer across the cell membrane more readily.[45] While the primary action of anesthetics involves interaction at the lipid bilayer and some effects of anesthetics on cellular activity are attributed to ability to perturb cell membrane phospholipid organization,[221] the effects of anesthetics on the lipid bilayer of the cell membrane are minimal.[48,222] Anesthetic activity has also been attributed to ability to perturb membrane protein conformation,[221] and several studies support that anesthetics primarily act directly on proteins rather than on lipids.[44] The role of second messengers on anesthetic action remains uncertain,[44,222] and other tissues may be affected by contact with lipid-soluble drugs in the absence of ligand gated receptors or changes in membrane protein conformation and interaction with cytoskeletal components, even if relatively little transport across the lipid bilayer occurs.[44,221-223]

During the cleavage stage in the preimplantation embryo, environmental insults to the embryo may result in embryonic death and miscarriage, or in some cases, intact survival.[4,122] This "all or none" result is

due to the largely undifferentiated cells of the early embryo, making repair possible through multiplication of the still omnipotent cells.[4] Even when survival is possible, non-lethal insults to the embryo may still have the ability to compromise development.[151,224] Physical or chemical insults have the potential to irreversibly damage a developing embryo and embryotoxicity may be influenced by numerous mechanisms.[225] Oxidative stress is a predominant contributor to developmental complications; oxidative stress has been found to cause a developmental block in early preimplantation embryos,[224] and exposure to oxidants during the first trimester is associated with an increased risk of congenital defects.[226] Oxidative stress is caused by an imbalance between ROS generation and antioxidant defense systems, which results in cell damage either directly or by altering cell signaling pathways[69,226] or a number of additional secondary processes. ROS may alter signal transduction, damage cellular macromolecules,[226] oxidatively cause DNA or RNA damage and affect repair, or increase lipid peroxidation, protein oxidation,[190,227] and ATP depletion.[224] Exogenous factors such as drug exposure and radiation may result in ROS production,[69,190,227] or cause and imbalance in antioxidant defense mechanisms in the embryo,[69,226] but ROS levels may also be elevated by endogenous factors such as increased mitochondrial respiration or inflammation and infection.[69] Free oxygen or nitrogen radicals can also damage mitochondrial membranes, which can have an autocatalytic effect in which oxidative stress is intensified by generation of new sources of ROS.[198] This damage is capable of inducing a developmental block,[224] as well as causing apoptosis or necrosis.[190] Once free radical damage is initiated, this damage can become autocatalytic; for example by damaging mitochondrial membranes new sources of ROS may be generated.[198]

Expression of approximately 10,000 genes must be expressed in a well-orchestrated manner for regular development of the preimplantation embryo and early fetus,[228] and exposure to teratogens may directly or indirectly perturb the expression of genes critical for normal development.[229] Chemical insults and oxidative stress are capable of causing DNA or RNA damage,[190,227] which may affect a number of embryonic genes depending on the timing of exposure. Prior to ZGA, oxidative stress to the embryo has been found to cause permanent developmental arrest.[190] Mutations of maternal-effect genes or interference with embryonic genome activation and transcription have the potential to result in arrested or delayed embryonic differentiation. A number of embryonic genes with maternal effects have been identified (*Mater, Hsf1, Zar1, Basonuclin1*), and mutation of maternal-effect genes has been found to induce partial or total developmental arrest in embryos at different developmental stages (e.g. in 1-cell *Hsf1* and 2-cell *Mater*).[197] Failure to activate the embryonic genome has also been found to arrest preimplantation development.[170,177] The preimplantation embryo is particularly sensitive to mutagenic agents or oxidative stress during ZGA,[190,198] and any condition which enhances ROS accumulation during ZGA may be detrimental.[190] Under normal conditions, ROS increase has been found to culminate during ZGA, suggesting that during this transition, oxidant defense is depleted and must be replenished by ZGA.[190] This relatively weak antioxidant defense in the early embryonic stages[227] often results in defective development when exposed to oxidative stress from exogenous agents.[224]

Culture conditions can alter the rate of degradation of mRNAs, and the degraded products can affect methylation and gene expression.[228] Alteration of the methylation pattern can lead to silencing or induction of a specific gene, and is a primary candidate for gene expression deviations.[228] Extracellular conditions may also cause signal transduction pathways target transcription factors, transcriptional coregulators and chromatin-modifying factors, leading to their phosphorylation by protein kinases or dephosphorylation by protein phosphatases.[230] These modifications facilitate gene expression by directly regulating distinct aspects of transcription factor function, including cellular localization, protein stability, protein-protein interactions, and DNA binding.[230] Any teratogen-influenced alteration during chromatin remodeling could cause incomplete reprogramming of transcription factor genes and result in abnormal gene expression.[202] Abnormal gene expression can affect a number of critical processes including transcription, mRNA processing, oxidative phosphorylation, metabolism, transmembrane solute transport, or protein biosynthesis, modification and degradation.[231]

The embryonic cell cycle can be modified by introduction of new components, substrates, or pathways, or altered interactions between them.[232] The precise cell cycle stage during which the development of embryos will arrest or delay coincides with initiation early embryonic gene expression,[198] and at different phases of the cell cycle, the embryo may be more resistant or sensitive to environmental factors.[15] During the G1 phase of the cell cycle, preimplantation embryos are particularly susceptible to environmental stress.[15] The timing of teratogen exposure during the cell cycle is thus very important to embryonic survival. In the mouse embryo, mitosis of the first cleavage division and the G1 phase of the second cell cycle has previously been found to start 16-18h after insemination and reach 90% by 22-24h.[233] First blastomeres of 2-cell embryos complete DNA synthesis 20-22h after insemination and by 28-30h 80% of blastomeres emerged from this phase.[233] The G2 phase lasts 12-15h in 2-cell embryos, and similar to the other phases of the cell cycle, is reduced sharply in subsequent cycles.[6,233,234] Since the sensitive G1 phase is much longer in 2-cell embryos than subsequent cell cycles, the second cleavage division may be more susceptible to environmental factors than subsequent divisions.[6,233,234]

Cytotoxic agents have the potential to prevent cell division by disrupting the mitotic spindle,[6] and antimitotic effects may be exerted directly or indirectly by oxidative stress. Antimitotic effects may be produced by destruction of the spindle and impaired chromatid separation during mitosis, or by the inactivation of Cdc25 phosphatases. During mitosis, improper attachment of kinetochores to microtubules triggers the spindle assembly checkpoint (SAC), which regulates progression of mitosis, preventing the onset of anaphase and potential incorrect segregation of the genetic material into daughter cells.[210] The local anesthetics dibucaine, tetracaine, procaine, and lidocaine have been found to significantly affect the organization of cytoskeletal components and their plasma membrane attachment points.[223] Electron microscopy found evidence of structural alterations in microtubule and microfilament organization 3T3 cells treated with these anesthetics, and the effects of these local anesthetics were duplicated by treating cells with colchicine (a microtubule polymerization inhibitor) together with cytochalasin B (a

microfilament formation blocker).[223] Other cell cycle control mechanisms such as Cdc25 phosphatases may be affected by anesthetic exposure. Cdc25 phosphatases are cell cycle control proteins,[235] essential as checkpoint response mediators under normal conditions, and in response to DNA or oxidative damage.[235, 236] When DNA is damaged or incompletely replicated, Chk1 and Chk2 checkpoint kinases inactivate Cdc25 phosphatases to halt cell cycle progression.[237] ROS catalyzed Cdc25 phosphatase inactivation have also been found to lead to a block of the cell cycle progression.[236]

Mitochondria are responsible for many processes critical to the initiation and progression of early development,[190] such as oxidative phosphorylation and provision of intermediates for glycolysis; as well as affecting ion homeostasis, participating in amino acid metabolism, signal transduction, and apoptosis.[196] Mitochondria also regulate the redox-balance;[197] however if free radicals damage mitochondrial membranes, many processes can be disrupted and new sources of harmful ROS may also be generated.[198] Apoptosis is necessary to early embryo survival, and the first apoptosis in the preimplantation embryo may take place as early as during the activation of the embryonic genome, however apoptosis may have detrimental effects including embryonic arrest if the number of apoptotic cells is elevated,[178] or if the balance of pro-apoptotic and anti-apoptotic proteins is affected.[201] In vitro causes of apoptosis may be attributed to suboptimal culture conditions, lack of survival factors such as growth factors, chromosomal abnormalities, or excessive reactive ROS production.[178] In preimplantation embryos in vitro, cells that begin to undergo or have died by apoptosis display characteristic visible cellular and nuclear fragmentation, as well as DNA fragmentation which can be detected by a number of methods.[238]

Since elevated apoptosis has previously been detected in fragmenting preimplantation embryos,[238] consideration of the potential induction of apoptosis by environmental factors is necessary. In vitro study of neurons differentiated from human embryonic stem cells found ketamine to increase ROS production and induce differential expression of oxidative stress-related genes; as well as dose- and time-dependently cause cell death.[103] Ketamine was also found to induce mitochondrial ultrastructural abnormalities, decrease inner mitochondrial membrane potential ($\Delta\Psi m$), and increase cytochrome c release from mitochondria.[103] The consequences of cytochrome c release depend on the cell type, but cytochrome c is a caspase activator and is essential part of the apoptosome, which activates caspase-9 and then activates other caspases to orchestrate apoptosis.[173] In cells containing large amounts of endogenous caspase inhibitors, cytochrome c release may be down regulated, resulting in failure to induce apoptosis and the eventual loss of electron chain transport, which can contribute to necrosis.[173]

Like ketamine, other general anesthetics such as pentobarbital or propofol may also have profound effects on ROS and mitochondrial function, however further investigation is necessary. Future study to compare the effects of ROS in somatic cells with those of the preimplantation embryo would also have significant implications. Pentobarbital has been found to suppress ROS generation and increase endogenous antioxidant ability in the rat hippocampus,[239] but at high concentrations, pentobarbital was found to be unable to protect hepatocytes from hydrogen peroxide induced oxidative stress; rather it

intensified stress damage.[240] Pentobarbital was also unable to significantly decrease oxidative stress-induced apoptotic and necrotic cells, however under the same conditions, propofol showed an ability to decrease the number oxidative stress-induced apoptotic and necrotic cells.[240]

Similar to several phenol-based antioxidants, propofol has been found to possess free radical scavenging properties,[241] as well as anti-inflammatory effects in vivo.[242] Though propofol possesses anti-apoptotic properties, stimulation of in vivo enzymatic systems such as the heme oxygenase (HO) pathway is important to this protective effect.[69] In a study investigating the potential protective effects of propofol, dosages were shown to decrease ROS-mediated caspase-3 activation and DNA damage in hepatocytes by stimulation of the HO pathway.[69] Additional in vivo study of propofol effects have also found it to reduce inflammatory responses in macrophages by inhibiting ROS-regulated Akt/IKKβ/NF-κB signaling,[242] as well as to protect against mitochondrial dysfunction and decrease production of mitochondrial ROS following induction of cerebral ischemia-reperfusion.[243] The protection against mitochondrial dysfunction was suggested to be the result of inhibition of mitochondrial membrane permeability and the reduction mitochondrial ROS production,[243] however the protective effect of propofol is uncertain.[69] In vitro effects of propofol require further investigation, most of the protective effects of propofol in response to ROS have been shown to result from stimulation of physiological pathways absent in the preimplantation embryo.[69,242]

Metabolism, nutrient uptake, and water transport play a critical role in the development of the embryo, and in addition to oxidative damage to mitochondrial membranes, metabolism may be altered or damaged in many ways.[198] Glucose uptake is a critical process in the embryo, being especially important during later stages of preimplantation development.[48] Uptake of glucose has previously been found to be interrupted by anesthetics,[48,244] and interference with facilitators of glucose uptake has also been shown to result in a high rate of apoptosis in murine blastocysts.[244] General anesthetics pentobarbital, ketamine, etomidate, and propofol have previously been compared for effects on and interactions with GLUT1 in murine fibroblasts (which predominately express GLUT1).[48] Pentobarbital and ketamine were found to interact directly with GLUT1; and all four compounds inhibited glucose transport.[48] The inhibition of GLUT1 and associated decreased glucose transport previously found to be induced by pentobarbital and ketamine may also have a similar effect on the mouse embryo. Since GLUT1 is expressed throughout preimplantation development, extended exposure to anesthetics may suppress adequate glucose transport to either delay or altogether arrest differentiation. Although glucose is not utilized as the primary energy substrate of the 2-cell embryo,[179,188,189] glucose uptake has been detected during the first several cleavage divisions.[179,193]

Glucose transporter inhibition can increase cellular apoptosis in the preimplantation embryo,[244] which may produce detrimental effects such as arrested differentiation.[178] Inhibition of GLUT1 expression in mouse blastocysts has previously been found to significantly lower the function of GLUT1, resulting decreased glucose transport and hyperglycemia.[244] Apoptosis was initiated in response to the hyperglycemia, which was found to act as a cell death signal triggering a BAX-dependent apoptotic cascade.[244] The result of this study supported the assumption that increased apoptosis at the blastocyst

stage due to maternal hyperglycemia may result in loss of key progenitor cells and manifest as a resorption, malformation, or if a significant cell loss occurs, as a miscarriage,[244] an adverse pregnancy outcome with elevated risks in diabetic women.[245] Increased apoptosis during early embryonic development may also contribute to numerous manifestations of developmental defects following anesthetic exposure,[2,6,7-15,116] as well as congenital malformations associated with barbiturate (phenobarbital) exposure during pregnancy, such as midface and digit hypoplasia,[219] and microcephaly;[216,219] however further study of necessary to support any significant correlation.

Teratogens can affect the preimplantation embryo by directly altering cellular processes or increasing oxidative stress,[225] and since the early embryo has limited regenerative capacity,[4] chemical or oxidative insults may cause irreparable damage. Due to relatively weak antioxidant defense especially during early stages,[227] stress from environmental insults often results in defective development.[224] Multiple mechanisms are present in the preimplantation embryo to protect against exogenous compounds and ROS, however the activity of these responses varies during development. During the final stages of maturation, the oocyte stockpiles antioxidant defense against oxidant insults which may arise in the embryo during early development.[190,224] Endogenous protection against ROS within the embryo is served by antioxidant enzymes superoxide dismutase, glutathione peroxidase and γ-glutamylcysteine synthetase.[224] In addition, non-enzymatic antioxidants such as hypotaurine, taurine, ascorbic acid are present in follicular and oviductal fluids in vivo.[224] In vitro embryo culture media often contains antioxidants such as pyruvate and albumin (Appendix 1) to compensate for absence of in vivo protective mechanisms, since follicular and oviductal non-enzymatic antioxidants are not present in vitro.[224]

Additional resistance to environmental insults may be attributed to processes occurring during ZGA, and this resistance may be dependent upon the activation of the embryonic genome.[182] Previous study of propofol effects on zygotes and 2-cell embryos in vitro found differences in sensitivity between the two, suggesting resistance to environmental may vary at different stages even during early development.[15] This was thought to be the result of differences in stages of the cell cycle during the timing of initial exposure,[15] but may have also been the result of the development of a transcriptionally repressive state within the 2-cell embryos, which has been suggested to develop in the 2-cell embryo.[182] This repressive state may involve some aspect of chromatin remodeling coupled to the second round of DNA replication,[182] causing the 2-cell embryo to be more resistant to nuclear degradation since modifications such as DNA methylation and histone deacetylation mask internucleosomal sites for DNA cleavage.[202]

Another cellular response to oxidative stress or chemical insult is increased expression of cell stress proteins such as heat shock proteins (HSPs).[225] HSPs[225] and stress-inducible genes[170,246] are regulated by heat shock factors,[225] some of first transcription factors expressed in the mouse preimplantation embryo.[170,225] Heat Shock Factor 1 (HSF1) is a major transactivator of stress-inducible genes in response to environmental changes,[246] and HFS1 function is required by early embryos for defense against oxidant insults and maintenance of redox-homeostasis and mitochondrial function.[197] Loss of HSF1 function

results in developmental arrest as early as the 1- to 2-cell stage in embryos expressing the *Hsf1*$^{-/-}$ trait,[197,225,246] perhaps due to the fact that several HSP genes are down-regulated in *Hsf1*$^{-/-}$ oocytes.[197] HSF1 is not necessary to initiate zygotic transcriptional activity, however; *Hsf1*$^{-/-}$ zygotes have been found to express HSPs, showing that zygotic transcriptional activity can begin in the absence of HFS1 and its protective effects.[246] Hsp70.1 is one of the earliest genes constitutively expressed in the early embryo beginning with the onset of embryonic transcription.[228] Induced expression of HSPs due to suboptimal culture conditions and constitutive HSP expression may both represent essential mechanisms for successful embryo growth in an adverse environment, but inducible expression of Hsp70.1 is delayed until the morula or early blastocyst,[178] offering little protection in earlier developmental stages.

Specific Aims

The focus of this study was to evaluate the toxicity of IV general anesthetics to the preimplantation embryo. Potentially teratogenic agents may contact the early embryo in a number of ways including elective or emergency surgery, assisted reproduction procedures, or drug abuse, necessitating the investigation of possible deleterious effects during preimplantation development. Numerous studies document the potential detrimental effects to development following exposure to alcohol and anesthetics.[2,6,7-15,116,117] General anesthetics were selected as representatives for their mechanism of action, with additional consideration to the potential for abuse and prevalence of use in humans and laboratory animals. The primary targets of IV general anesthetics are CNS ion channel-linked receptors for the either glutamate (NMDA receptors), or GABA ($GABA_A$ receptors).[21] The $GABA_A$ receptor is the primary target of a diverse group pharmacological agents, including barbiturates, benzodiazepines, and general anesthetics halothane and propofol.[48,50,58-60] Agents such as ketamine HCl,[63] phencyclidine (PCP),[11,64] dextromethorphan,[65] and nitrous oxide[66] antagonize the binding of glutamate[11,63-66] by blockade of NMDA receptors to achieve anesthetic effects.[19] Examples of common injectable anesthetics used for laboratory animals and in veterinary medicine include pentobarbital,[68,88] ketamine, and propofol,[82] however these agents are also used for human anesthesia.

A majority of drug-abusing women are of childbearing age; consequently it is not unusual to encounter pregnant women who abuse illicit drugs, either due to negligence or disregard to the potentially harmful effects of this behavior.[114] Many studies found to refute the deleterious potential of drug exposure only investigate effects of short term exposure, and do not test binge conditions under which an abuser may expose an embryo to high concentrations for extended durations.[122] Pentobarbital has a relatively high potential for abuse for the same sedative/hypnotic effects for which it is clinically implemented.[115] The more widespread use of ketamine increases its availability, which in combination with its psychomimetic effects[28,31,32] may influence its even higher potential for abuse.[7,11,29,101] The abuse potential for propofol has not been completely defined, but its abuse is not uncommon among people working in the medical field and in certain areas of the world where celebrities have popularized its misuse.[70,135]

The perfect injectable anesthetic is yet to be developed,[82] but propofol is currently the most ideal anesthetic agent for TIVA.[20] Propofol is also a commonly used drug for IVF,[10,37,39,204,205] and since it has been detected in the follicular fluid of patients,[2,204] it has demonstrated an ability to make contact with an oocyte or an early embryo in uterine fluids. In recent years, the shortage of drugs such as propofol and thiopental has made the choice of an induction agent be more influenced by availability, [21] requiring investigation of alternative agents. While some of the emergence reactions of ketamine are undesirable,[28] its versatility,[21,26] positive hemodynamic profile,[23,29] analgesic effects,[73] and low cost[19,27,29] contributes to its continued use in humans,[19] and the use of ketamine is widespread for veterinary anesthesia.[82]

The development of other anesthetic agents such as propofol[24] in response to some of the negative anesthetic properties of barbiturates has led to the reduction of the anesthetic use of barbiturates,[15,23,30,33-43] however the long history of barbiturate use and research, as well as utilization for selective procedures or conditions, and potential for abuse compels continued study of this class of drugs. Pentobarbital anesthesia remains prevalent in many undeveloped countries,[82] and additional use of pentobarbital continues in the first-world as a second or third-line agent for a number of applications[24,73,82,84-87] Pentobarbital is currently approved by the FDA,[247] and its economic benefits and the promising potential of its stereoisomers may contribute to future widespread use. The decades of use of pentobarbital, in addition to its generalized availability, low cost, relatively rapid onset of anesthesia, and ease of injection to a variety of animals contributes to its continued widespread use in laboratory animals and in veterinary medicine,[68,82,85,88] and its use is especially prevalent in rodents.[68,88] Few barbiturates are currently used, however pentobarbital and thiopental are common agents in veterinary medicine, and the availability issues of thiopental[82] influenced the selection of sodium pentobarbital as a representative of barbiturates for this study.

The study of the preimplantation embryo is important to understand the mechanisms of embryotoxicity during early pregnancy.[138] The in vitro culture embryo assay is a valuable model for the assessment of the safety of drugs[155] and to screen potential toxic effects of pharmacologic agents which may come in contact with the egg or early embryo.[10,138] The preimplantation mouse embryo in vitro development bioassay elucidates the effect of a pharmacologic agent on cellular differentiation;[138] and the early embryonic stages provide indications of potential induction of fetal and postnatal defects.[157] Extended drug exposure periods are necessary in this system since some components causing failure of the bioassay may compromise embryo development, without being outright lethal.[151] Later development may also be assessed during extended exposure since components have the ability to affect the development of the inner cell mass directly.[151] The potential of embryos developing into blastocysts may relate to factors within the embryos which may be affected by contact with exogenous compounds,[152] and the timing during which development may be interrupted or delayed is necessary to establishing mechanisms by which effects are observed. Extended exposure may also assist in evaluation of potential embryotoxicity of pharmaceutical agents subject to habitual abuse.

The window of the preimplantational period, and the dosage and severity of embryotoxic effects which

may be exerted by sodium pentobarbital, ketamine HCl, or propofol has not been established. To observe potential adverse effects of these drugs, preimplantation 2-cell mouse embryos were cultured in three separate studies of 72h extended exposure. The objective of this study was to establish the window of preimplantation development during which each anesthetic agent affects cleavage through the normal progression of in vitro embryonic development, the concentration at which inhibitory effects are significant, and the extent to which this damage may be exerted. Embryos were exposed to incremental concentrations of sodium pentobarbital, ketamine HCl, or propofol, and development was monitored and evaluated. Incremental concentrations of these agents exceeding normal clinical concentrations found in follicular fluids and surpassing the normal clinical time of exposure were used to assess embryotoxicity for this study. Embryotoxicity was evaluated by analysis of the morphology, early cleavage, and subsequent differentiation of preimplantation mouse embryos subjected to prolonged exposure of sodium pentobarbital, ketamine HCl, or propofol from the 2-cell stage until blastocyst formation at the 72h endpoint. In the mouse model, blastocyst morphology provides a good indicator of embryonic potential for implantation, and the role of blastocysts hatching from the zona pellucida has been shown to be more important to this potential than the number or volume of cells.[152] Embryonic manifestations of abnormal appearance with evidence of fragmentation (appearance of fragments of cells within an embryo that are smaller than normal-sized blastomeres),[238] granulation, and degeneration of blastomeres were indications of embryotoxic and clastogenic damage, potentially contributing to delayed and inhibited differentiation or complete arrest of development.

CHAPTER 2

PROPOFOL

INTRODUCTION

The potential detrimental effects to development following exposure to alcohol and anesthetics is well documented,[2,6,7-15,116,117] however exposure is sometimes unavoidable; as many as 2% of pregnant women require surgery.[3] Exposure to harmful agents may also occur as a result of substance abuse during pregnancy.[116] Investigation of xenobiotics which could come in contact with an oocyte or early embryo is necessary, exogenous compounds may be potentially detrimental to fertilization, embryonic cleavage and differentiation, and subsequent development.[1] Propofol is a short-acting IV anesthetic, advantageous for its rapid action with short and predictable duration, with rapid, antiemetic, clear-headed recovery.[15,23,30,33-43] Propofol is highly lipophilic,[43,67] non-chiral,[68-70] and chemically distinct from all other IV induction agents.[70] Since its development, propofol has been widely accepted as the most ideal agent for TIVA as a general anesthetic,[20] and propofol TIVA has numerous advantages over anesthetic maintenance with volatile agents[19] for human and veterinary anesthesia.[82] Propofol is a frequent choice for sedation in monitored anesthesia care, as well as for induction and maintenance of general anesthesia[19] for gastrointestinal endoscopic procedures and minor surgical interventions,[37] for long-term infusions during surgery and on the ICU since it does not significantly accumulate,[26,30] and its rapid, short-acting effects make propofol a preferred agent for assisted reproduction procedures.[10,15,37,39,75,204,205] The abuse potential for propofol has not been completely defined,[70] however its mild euphoric and hallucinogenic effects makes the recreational use of propofol desirable.[135] Abuse of propofol is also thought to be higher among anesthesiologists and medical technicians since its widespread use in clinical practice makes propofol available, and concealment of abuse is easy due to its short duration of action.[70]

When used during transvaginal oocyte retrieval, propofol has been detected in follicular fluid[10,39,205] and has been found to accumulate at levels proportional to the total dose.[37] Previous studies have shown that even low concentrations of propofol during short-term exposure have the potential to interfere with normal fertilization, as well as being deleterious to preimplantation cleavage.[10,15] In a study to assess the ability of mouse oocytes to fertilize and subsequently develop to the blastocyst stage following 30 minute in vitro exposure to concentrations ranging from 50ng/mL to 5.0μg/mL, propofol was found to have significant effects.[10] When fertilization occurred, subsequent embryonic cleavage and development to the blastocyst stage was significantly impaired, and propofol was also found to result in parthenogenetic activation of cumulus-enclosed oocytes.[10] Propofol has been shown to negatively affect the ability of oocytes to fuse with spermatozoa without interfering with post-fusion events such as sperm decondensation and cell cycle resumption following post-fertilization exposure, however propofol may also inhibit zygotic

differentiation.[15] Comparison of 14h exposure to propofol concentrations ranging from 0.01 to 10μg/mL in zygotes and 2-cell embryos found inhibition of cleavage to the blastocyst stage in zygotes, however even at 10μg/mL, subsequent cleavage of 2-cell embryos was not significantly impaired.[15] This effect was thought to be a result of cell cycle differences at the time of exposure between zygotes and 2-cell embryos; cells at the G1 phase of the cell cycle are particularly susceptible to environmental factors, and the G1 phase is much longer in zygotes than in 2-cell embryos,[15,210] leading to a prolonged period of sensitivity in the 1-cell embryos.[15]

Although propofol has no known teratogenic effect in humans,[248] it carries and undetermined FDA teratogenic risk rating,[136] and has the ability to cross the placenta.[67,165] No evidence of teratological effects in rat and rabbit at lower doses ranging from 5-15mg/kg,[43] however neural development has been found to be significantly affected by higher doses.[211,249] Propofol at 25mg/kg has been found to induce complex changes in the developing rat brain, accompanied by age-dependent neurodegeneration in the cortex and thalamus at postnatal day 7,[211] and even 25% anesthetic effective dose (ED) (200mg/kg) has been found to induce neuroapoptosis in the developing mouse brain.[249]

Short-term exposure of early mouse embryos to propofol has been found to produce an adverse effect on embryonic development;[10,15] but extended exposure to the preimplantation embryo and effects on later embryonic and fetal development have yet to be determined.[35,36] The mouse system is valuable for screening the potential toxic effects of any xenobiotic compound which may come in contact with a developing embryo,[138] and the mouse embryo growth and development 72h bioassay is considered to be by the clinical reproductive industry the preferred assay for quality assurance/quality control (QA/QC) determination and validation of gamete and embryo research.[153] The window during preimplantation development, and the dosage and severity of embryotoxic effects which may be exerted by propofol has not been previously evaluated. Early embryonic stages provide indications of potential induction of fetal and postnatal defects,[157] and extended exposure allows determination of effects of later preimplantation development since some components have the ability to affect the development of the blastocyst and inner cell mass directly.[151] Evaluation of extended drug exposure is necessary since some components causing failure of the bioassay may compromise embryo development without being outright lethal.[151] Extended exposure may also assist in evaluation of potential embryotoxicity of pharmaceutical agents subject prolonged use or habitual abuse.

This study was performed with the objective of evaluating the potential of propofol to impair cleavage through the normal progression of in vitro embryonic development from the 2-cell to the blastocyst stage, and the developmental timing and extent to which damage may be exerted. Potential adverse effects of propofol were assessed by individual culture of preimplantation 2-cell murine embryos with incremental concentrations of propofol, starting with normal clinical concentrations and surpassing the normal clinical time of exposure. Initial experimentation showed extreme embryotoxicity at concentrations between 10-25μg/mL, well below the murine 50-200mg/kg[75,250] (50-200μg/mL) anesthetic induction ED. Inhibitory

effects were detected even at concentrations similar to the human ED dose range of 1.5-2.5mg/kg (1.5-2.5μg/mL)[19,26,30,43,67,74] during propofol anesthesia. Concentrations were modified during follow-up experimentation to 1.0μg/mL increments between 1-10.0μg/mL, and then further refined to 0.5μg/mL increments from 0.5-5.0μg/mL. Embryotoxicity was assessed by analysis of early cleavage and subsequent differentiation of 2-cell preimplantation embryos subjected in vitro to prolonged 72h exposure of propofol. Detection of abnormal embryo morphology including fragmentation, granulation, and degeneration of blastomeres provided indications of deleterious clastogenic damage capable of significantly inhibiting in vitro development.

METHODS

Animals

Embryos used in the study were produced on Institutional Animal Care and Use Committee (IACUC) protocol #12-017. Embryos were obtained from female mice 6-18 weeks old (B_6CBAF_1F, purchased from Jackson Laboratories, Bar Harbor, Maine) crossed with outbred CD-1 males 2-10 months old (Harlan Lab, Indianapolis, IN). This F2 hybrid mouse strain has proved to produce consistently high numbers of embryos with 100% development to blastula stage in 72h if all culturing variables are at an optimum. Mice were 4-6 weeks old when received and acclimated to the animal facility for at least 48h before use and were used within 12 weeks of purchase. Mice were maintained on a lighting regime of 14h light, 10h dark at 21°-25°C and given water and laboratory rodent chow (Harlan) *ad libitum*. Euthanasia was performed by cervical dislocation, approved by the Panel on Euthanasia of the American Veterinary Medical Association.

Superovulation And Mating

Female mice were selected at random for exogenously driven superovulation. Superovulation was achieved by intraperitoneal (IP) injection of gonadotropic hormones. IP injection was performed through the lower right quadrant of the abdomen using a 1mL syringe with a ½ inch 30 gauge needle. Beginning four days prior to use, the injection cycle consisted of two injections separated by 48h. The first injection at 0h was 0.1mL of a 50 international units (IU)/mL (5 IU) of pregnant mare serum gonadotropin (PMSG) (SIGMA, G4877, 106K1171; St. Louis) in phosphate buffered saline (PBS). At 48h after PMSG injection, 0.1mL of a 50 IU/mL (5 IU) solution of human chorionic gonadotropin (hCG) (SIGMA, CG5, 075K1442; St. Louis) in PBS was administered. At the time of hCG injection, each female was caged with one proven fertile male CD-1 mouse. Female mice go into estrous and mate within 6h of hCG injection, with ovulation occurring between 10 and 12h after hCG injection. Between 8 AM and 10 AM (16h after hCG injection) the following morning, female mice were inspected for vaginal copulatory plugs and the result of the performance of each male was recorded. At this time mated and unmated females were segregated and marked appropriately with cage cards; only mated animals were used. Unmated animals were submitted to the same injection cycle after a two-week recovery period during which the pituitary gland is less sensitive

to gonadotropins. Females cycled three times without successful mating were removed and euthanized by cervical dislocation.

Collection Of Embryos

Females with vaginal copulatory plugs were euthanized using cervical dislocation starting approximately 42h after the hCG injection (pregnancy day 0) and immediately prior to embryo collection. The abdomen of the mouse was entered by producing a small hole above the umbilicus then retracting the skin simultaneously down over the hind legs and over the forelegs in a process known as de-gloving. This technique leaves a sterile field for entry without any mouse hair and obviates use of antiseptic agents that may compromise embryo viability. Bilateral celiotomy was performed to expose the oviducts of pregnant animals. Salpingectomy was performed by gently stretching and tearing the mesosalpinx and mesometrium away from each oviduct and uterine horn, and then cutting between the oviduct and ovary at the cephalad end and the oviduct and the intramural junction at the caudal end with microsurgical scissors. The two oviducts were then placed in each sterile 35 x 10mm polystyrene culture dish (Nalge Nunc International) containing 2mL of modified Krebs-Ringer-bicarbonate (mKRB) medium (See Appendix 1). A Zeiss dissecting stereomicroscope (Eastern Microscope Co, Raleigh, N.C.) at 20x magnification, watchmaker forceps (Dumont and Fils, Switzerland) was used to slide the fimbriated end of the oviduct onto a 30-gauge (FineJect, Henke Sass Wolf) sterile needle attached to a 1 mL sterile syringe (Norm-Ject). Embryos were irrigated from the oviducts with medium lightly expressed from the syringe. Each animal can be expected to produce an average of 15 embryos per successful mating (successful mating is approximately 1 per 2 stimulated females = (½)).

Embryos

Individual morphologically normal (judged by oval, bilateral symmetry of the two blastomeres with narrow perivitelline space and presence of two small polar bodies) 2-cell embryos was evaluated at 32x magnification and collected from the petri dish with a 10µL micropipette (Eppendorf) fitted with a sterile tip (Avant) and aseptically dispensed into 96 well U-bottom culture plates (Falcon, 35-3077) with one embryo per well. Embryos were cultured in a NuAire Autoflow IR Water Jacketed CO_2 Incubator at 37°C, 5% CO_2 for 72h. Differentiation was evaluated every 24h at 40x and 100x using a Nikon Diaphot inverted microscope. Each test medium was evaluated in triplicate cultures.

Solutions And Media

Media used was modified Kreb's ringer solution (mKRB) supplemented with 4mg bovine serum albumin (BSA) per mL (See Appendix 1) and conditioned at 37°C 18-24h. Propofol (2-6-Diisopropylphenol 97%, Sigma Aldrich D126608-5G) was dissolved in mKRB media at various concentrations and equilibrated in the CO2 incubator for at least 12h before use. The control solution for

the propofol experiments was mKRB media. Test concentrations of media containing propofol were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 15.0, and 25.0μg/mL.

Statistics

A 72 hour embryo culture endpoint with minimum development of 65% blastula and combined 80% morula + blastula embryo morphology is necessary to report a passing score on the mouse embryo assay.[251] Cell number and overall developmental stage of embryos in specific treatments were analyzed using one-factor fixed-effects randomized block analysis of variance (ANOVA) performed using IBM SPSS 20 to test for overall significance among treatments. This was followed by Tukey's HSD (honestly significant difference) test for differences between means of specific treatments. The Shapiro-Wilk test of normality and Levene's test of equality of variance were also used to evaluate whether samples were normally distributed and if there was statistically significant variation between treatments. The significant groups were then tested with χ^2 for homogeneity and difference of proportions. Culture of at least 15 embryos is performed with each item that we test to meet the CLIA '88 federal law to establish toxicity for the tested item. This embryo number (15) was established by the R.J. Swanson Mouse Embryo Laboratory as the standard for their testing protocol.

RESULTS

The results of propofol experiments are presented in Table 1 and Table 2. Preliminary testing included concentrations of 0.1, 1.0, 2.5, 5.0, 10.0, 15.0, and 25.0μg/mL propofol. All concentrations exceeding 5.0μg/mL propofol yielded 100% degeneration and fragmentation with arrest of cleavage at the 2-cell stage. Test concentrations were then modified to 1.0μg increments between 1.0-10.0μg/mL propofol to evaluate dosage range allowing differentiation. After development past the 2-blastomere stage was inhibited in 100% of embryos at all concentrations exceeding 5.0μg/mL, the dosage range was further refined to increments of 0.5μg/mL between 0.5-5.0μg/mL, to determine the range within which embryonic development was inhibited but not entirely arrested.

Propofol significantly induced degeneration at concentrations exceeding 2.0μg/mL (Table 2), and inhibition of differentiation to blastocyst was significant at concentrations above 1.5μg/mL. Degeneration and nuclear fragmentation was observed in 79% of embryos within the 5.0μg/mL propofol group ($\chi 2$, $p<0.001$). In addition, 87% of embryos displaying degeneration at this concentration were arrested at the 2-cell stage (Fig. 8), and only 21% were able to reach the blastocyst stage. At 4.5μg/mL, 17.6% of embryos reached the blastocyst stage, with 70.6% degenerating before reaching the morula stage, and 50% of degenerating embryos were arrested at the 2-cell stage (Fig. 8). At 4.0μg/mL, 88.5% of embryos exhibited fragmentation and degeneration, with 57.7% arrested at the 2-cell stage. The 3.5μg/mL concentration resulted in the least amount of variance between groups (developmental stage reached) of any

concentration tested (9.333); 50% of embryos at this concentration degenerated with 20% arrested at the 2-cell stage (Fig.7; Table 2), 20% reached the morula stage, and 30% reached the blastocyst stage.

At 3.0μg/mL propofol, 19.4% of embryos displayed degeneration; however of those degenerating, 86% were arrested at the 2-cell stage. 3.0μg/mL propofol also halted 25% of embryos at the morula stage, and allowed only 55.6% reached the blastocyst stage. At 2.5μg/mL propofol, 76% of embryos achieved the blastocyst stage. Those unable to reach blastocyst at 2.5μg/mL degenerated prior to the morula stage, resulting in a failing embryo score, though 50% of degenerating embryos completed at least one additional cleavage division (Table 2; Fig. 8). Below 2.5μg/mL, propofol did not significantly inhibit differentiation to at least the morula stage. This effect was especially evident in groups below 2.0μg/mL. In every group below 2.0μg/mL, the blastocyst stage was reached in over 80% of embryos, with less than 10% degenerating and/or fragmenting.

Results of the one-factor fixed-effects randomized block ANOVA for overall significance among treatments found the means of all treatment groups to be significantly different ($F=3.9831$; $df=3,56$; $P=0.012136$). Analysis of groups from 0μg/ml (control) to 5.0μg/ml propofol also found significantly different means ($F=6.555761$; $df=3,36$; $P=0.001189$), however a significant effect was not found between these lower concentrations. Analysis was followed by Post Hoc Tukey's HSD test to evaluate differences between specific treatments. The Tukey's HSD test did not reveal significant effect between specific blocks suggesting that lower concentrations did not significantly inhibited differentiation. The Shapiro-Wilk test of normality indicated that the samples were not normally distributed in control groups, 1.5μg/mL propofol, and 6.0-10.0μg/mL propofol ($P=0.047$, 0.00, 0.00). Levene's test of equality of variance was statistically significant ($P=0.002$), indicating a significant effect on differentiation between the concentrations tested.

Table 1. Propofol 72h effects on embryonic development: percentage of embryos reaching specific stage of differentiation during preliminary experimentation

Propofol μg/mL	2-Cell	4-Cell-12-Cell	Total Degenerating/ Fragmenting	Morula	Blastocyst	Total Cultured
0 (Control)	0%	0%	0%	0%	100%	10
0.1μg/mL	0%	10.0%	0%	0%	90.0%	10
1.0μg/mL	0%	0%	0%	0%	100%	10
2.5μg/mL	0%	16.7%	16.7%	0%	83.3%	12
5.0μg/mL	66.7%	25.0%	91.7%	0%	8.3%	12
10μg/mL	100%	0%	100%	0%	0%	10
15μg/mL	100%	0%	100%	0%	0%	16
25μg/mL	100 %	0%	100%	0%	0%	12

Propofol buffered with mKRB media+BSA was tested at the above concentrations to ascertain potential for embryotoxicity. A control solution containing mKRB+BSA embryo culture media, with no (0ng/mL) propofol was used. Degenerating refers to abnormal development or deterioration of the embryo before reaching the compact morula stage.

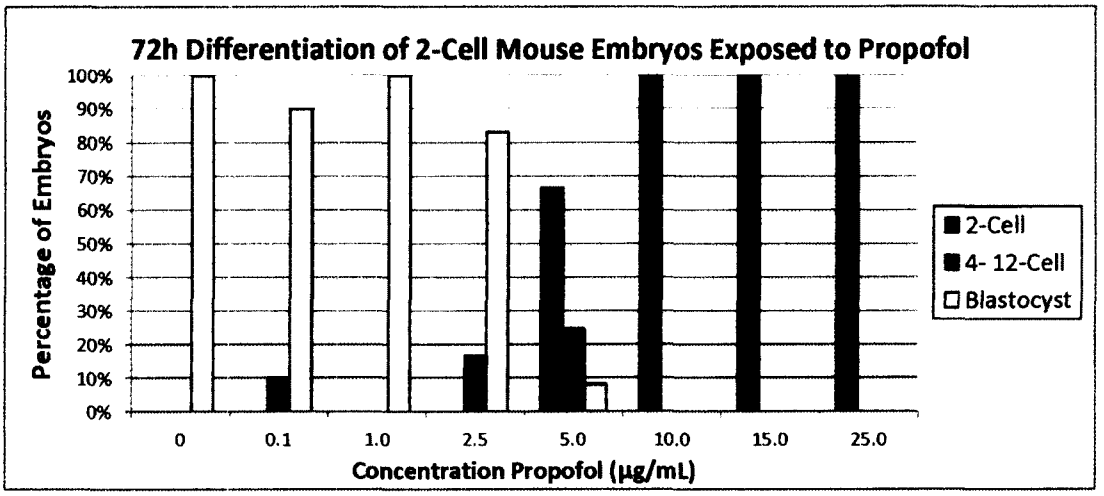

Fig. 6. 72h differentiation of 2-cell mouse embryos exposed to propofol: preliminary results. Percentage of embryos reaching specific stage of differentiation during preliminary experiments. Embryonic arrest prior to morula, or completed differentiation to blastocyst rendered reporting morula stage not applicable for preliminary studies (see Table 1). A control solution containing mKRB+BSA embryo culture media, sans propofol was used. All concentrations exceeding 5.0µg/mL were found to be 100% toxic to embryonic development.

Table 2. Propofol 72h effects on embryonic development: percentage of embryos reaching specific stage of differentiation

Propofol µg/mL	2-Cell	4-Cell-12-Cell	Total Degenerating	Morula	Blastocyst	Total Cultured
0 (Control)	5.0%	0%	5.0%	2.5%	92.5%	40
0.5µg/mL	4.8%	0%	4.8%	14.3%	80.9%	21
1.0µg/mL	0%	0%	0%	14.7%	85.3%	34
1.5µg/mL	0%	4.8%	4.8%	4.8%	90.4%	21
2.0µg/mL	2.9%	2.9%	5.7%	28.6%	65.7%	35
2.5µg/mL	12.0%	12.0%	24.0%	0%	76.0%	25
3.0µg/mL	16.7%	2.8%	19.4%	25.0%	55.6%	36
3.5µg/mL	20.0%	30.0%	50.0%	20.0%	30.0%	20
4.0µg/mL	57.7%	30.8%	88.5%	7.7%	3.8%	26
4.5µg/mL	35.3%	35.3%	70.6%	11.8%	17.6%	17
5.0µg/mL	68.9%	10.3%	79.2%	0%	20.8%	29
6.0µg/mL	100%	0%	100%	0%	0%	8
7.0µg/mL	100%	0%	100%	0%	0%	8
8.0µg/mL	100%	0%	100%	0%	0%	11
9.0µg/mL	100%	0%	100%	0%	0%	11
10.0µg/mL	100 %	0%	100%	0%	0%	10

Propofol buffered with mKRB media+BSA was tested at the above concentrations in follow up experiments. This phase was performed within range of inhibition of differentiation found by preliminary experimentation (Table 1) to ascertain potential for embryotoxicity. Control solution identical to preliminary experiments.

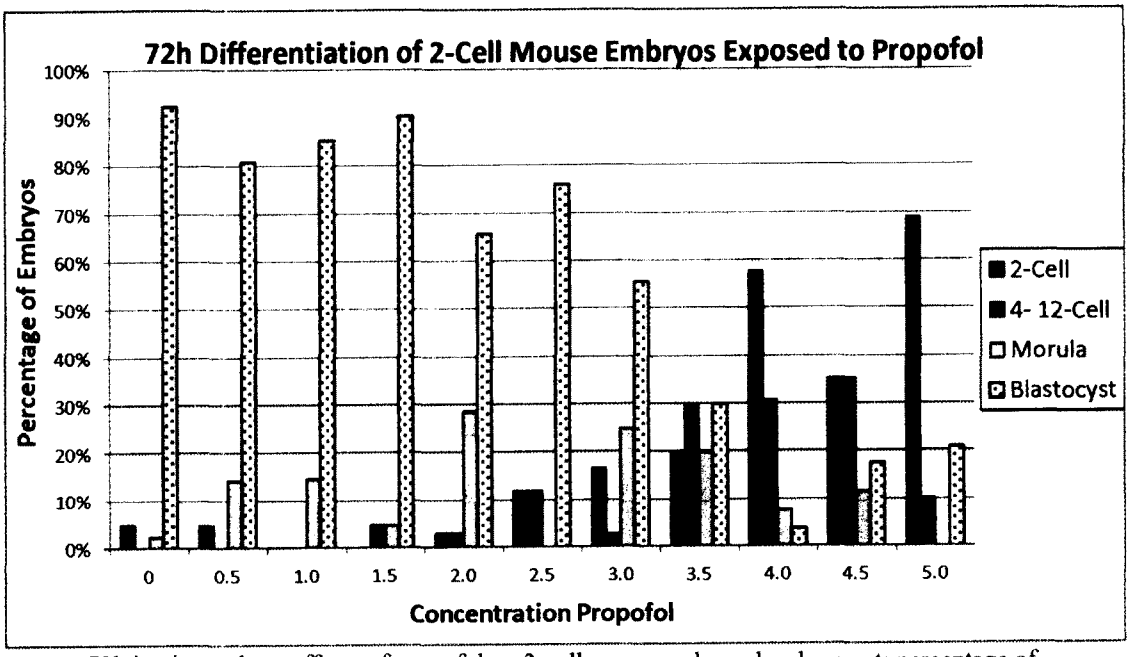

Fig. 7. 72h in vitro culture effects of propofol on 2-cell mouse embryo development: percentage of embryos reaching specific differentiation stages. A control solution containing mKRB+BSA culture media, sans propofol was used. Exposure to higher concentrations (6.0, 7.0, 8.0, 9.0, and 10.0µg/mL) was found to be 100% toxic to embryonic development. See Table 2.

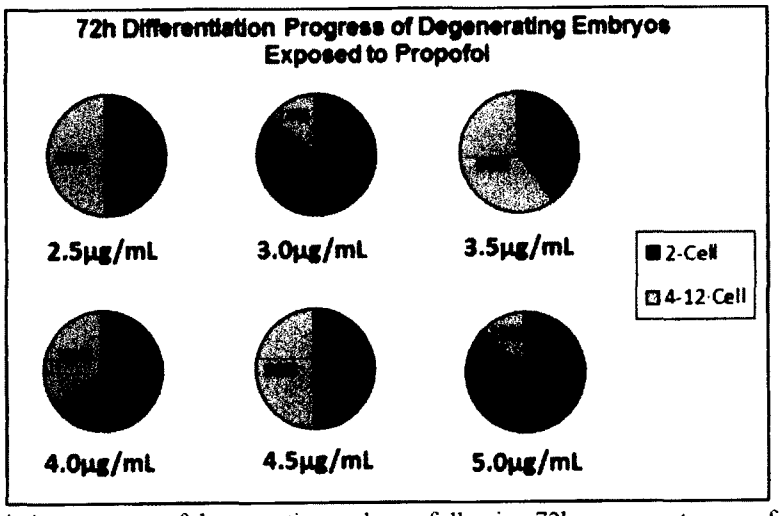

Fig. 8. Differentiation progress of degenerating embryos following 72h exposure to propofol. Percentages of embryos arrested at the 2-cell stage in comparison to embryos able to undergo at least one cleavage division before degenerating and/or fragmenting (4- 12-cell).

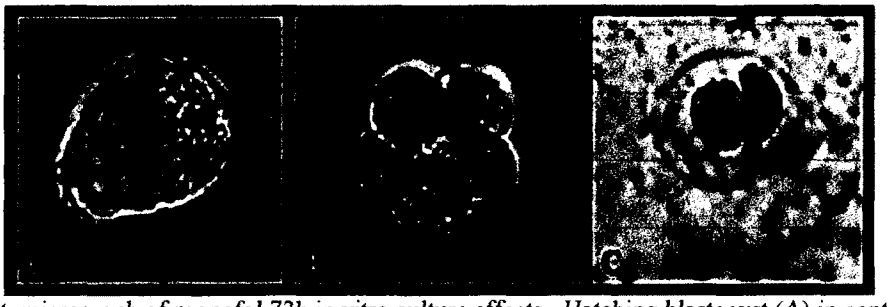

Fig. 9. Photomicrograph of propofol 72h in vitro culture effects. Hatching blastocyst (A) in control solution (mKRB media + BSA), 8-cell embryo (B) cultured at 4.0μg/mL propofol; arrested 2-cell embryo (C) cultured at 10.0μg/mL propofol. Photomicrograph taken at 200x using DIC Optics.

DISCUSSION

The results of this study suggest that extended propofol exposure produces dose-dependent inhibitory effects to the differentiation of preimplantation murine embryos in vitro. Propofol significantly decreased 72h differentiation to the blastocyst stage at the highest concentrations tested (6.0-10.0μg/mL propofol), with 100% of embryos within these groups degenerating at the 2-cell stage. Blastomeres of embryos within these treatment groups displayed substantial fragmentation indicative of apoptotic damage and no evidence of further differentiation was exhibited. Caution should be exercised when forming conclusions about degenerative effects though; it is not uncommon to find a few embryos fragmenting or degenerating, even in culture with Kreb's media preparations alone.[144]

Degeneration and nuclear fragmentation was significant at 5.0μg/mL propofol, where 68.9% of embryos exhibited a 2-cell block. 10.3% of embryos degenerating at 5.0μg/mL propofol were able to complete one cellular division before arresting at the 4-cell stage (Fig. 8); however all surviving embryos that did not degenerate at this concentration completed differentiation to blastocyst (20.8%). Significant inhibition of differentiation to the blastocyst stage was also observed between 2.0-4.5μg/mL, with an overall trend of increased inhibition at progressively rising concentrations. This generalization was deviated from by embryos cultured at 4.0μg/mL. At 4.0μg/mL, propofol exerted effects (89% degeneration) resembling those found at concentrations exceeding 5.0μg/mL (100% degeneration). However, like embryos cultured at 4.5μg/mL, cleavage was not entirely blocked at 4.0μg/mL and 35% of arrested embryos completed at least one mitotic division (Fig. 8).

By the 72h conclusion of the culture period, concentrations between 2.5-4.5μg/mL propofol resulted in significant variation in the stage of differentiation reached. Although 2-cell block was significant at concentrations above 2.0μg/mL, embryos degenerating at 2.5μg/mL and between 3.5-4.5μg/mL propofol were able to complete at least one cleavage division before fragmenting and cleavage arrest (Fig. 8). Later embryonal delay was significant in groups exposed to 2.0, 3.0, 3.5, and 4.5μg/mL propofol; at these concentrations, a significant percentage of embryos were halted at the morula stage and unable to undergo further differentiation to blastocyst. Embryos cultured at concentrations below 3.0μg/mL propofol

exhibited significant capacity for differentiation to at least the morula stage. This effect was especially evident in groups cultured at concentrations below 2.0μg/mL. Although a significant percentage of embryos exposed to 2.5μg/mL propofol reached the blastocyst stage, 24% degenerated to result in a failing embryo score. For human IVF, media is not recommended for use when less than 75% of the embryos develop to morula or blastocyst, or more than 15% appear to be degenerating or fragmenting after 72h culture,[144] but minimum development of 65% blastocyst and combined 80% morula + blastocyst embryo morphology is necessary to report a passing score on the mouse embryo assay.[251] Failing embryo scores were recorded for embryos cultured at all concentrations exceeding 2.5μg/mL propofol.

The embryotoxicity of propofol exposure in the current study may be attributed to a variety of biochemical mechanisms or a combination of deleterious effects. Exogenous compounds can affect development of the preimplantation embryo by increasing oxidative stress, and due to relatively weak antioxidant defense during early developmental stages,[227] this stress often results in defective development.[224] Oxidative stress has previously been found to produce a developmental block in mouse embryos cultured from the 2-cell stage,[224] and propofol-induced oxidative stress is a likely factor contributing to the observed fragmentation and inhibited embryonic differentiation at higher concentrations. Oxidative stress is also capable of affecting development via numerous secondary effects such as directly causing DNA or RNA damage,[190,227] as well as affecting nutrient uptake and metabolism,[198] mitosis,[236] and apoptosis.[178] Oxidative stress may be intensified by autocatalytic effects; free radicals can damage mitochondrial membranes and generate new sources of ROS.[198] Environmental agents that elevate levels of free radicals may also affect the developing embryo by increasing lipid peroxidation and protein oxidation.[227] Although propofol has been shown to exhibit antioxidant[69] and free radical scavenging properties,[241] previous results have shown many of the protective effects of propofol to be the result of stimulation of later developing enzymatic pathways such as the HO pathway,[69] or by reduction of macrophage inflammatory response.[242]

In the current study, the survival of embryos able to complete at least one cellular division, or of those exhibiting delayed differentiation suggests that at lower concentrations, embryos were resilient enough to stagger through at least one additional cleavage division before degenerating. This embryonic survival may be attributed to modest toxic effects at lower concentrations affecting few cellular processes, perhaps in combination with the capacity of embryonic endogenous mechanisms to preserve some normal functions in the presence of environmental insult. During the cleavage stage in the preimplantation embryo, environmental insults to the embryo may result in embryonic death or survival.[4] Even when survival is possible, development may still be compromised by non-lethal insults to the embryo.[151,224] The undifferentiated cells of the early embryo can enable repair through multiplication of the still omnipotent cells,[4] but because the embryo has limited regenerative capacity, oxidative injury may result in irreparable damage.[151,224]

A cellular response to oxidative stress or chemical insult is increased expression of cell stress proteins such as HSPs.[225] Induced expression of HSPs due to suboptimal culture conditions and constitutive HSP expression both represent an essential mechanism for successful embryo growth in an adverse environment.[178] Along with other stress-inducible genes, HSPs are regulated by heat shock factors, but loss of heat shock factor function has been shown to result in developmental arrest as early as the 2-cell stage.[170,197,225,246] Hsp70.1 is one of the earliest genes constitutively expressed in the early embryo beginning with the onset of embryonic transcription;[228] however inducible expression is delayed until the morula or early blastocyst.[178] Inadequate HSP expression may have contributed to inability of 2-cell embryos to recover from the chemical insult of exposure to greater than 4.5μg/mL propofol. HSP expression may have also assisted in the significant survival of embryos at 2.5-4.5μg/mL and lower concentrations.

If propofol exposure directly altered HSP transcripts or proteins, or oxidatively induced down-regulation of HSP expression, the intracellular redox-balance may have been affected.

Apoptosis is a necessary process in normal embryonic development, however apoptosis may have detrimental effects including embryonic arrest if the number of apoptotic cells is elevated,[178] or if the balance of pro-apoptotic and anti-apoptotic proteins is affected.[201] Aside from excessive ROS exposure, additional in vitro causes of apoptosis may be attributed to suboptimal culture conditions or chromosomal abnormalities.[178] High levels of apoptosis have previously been detected in fragmenting preimplantation embryos,[238] and the fragmentation of blastomeres observed in degenerating embryos in this study was indicative of apoptosis. Cellular death and fragmentation characteristic of apoptosis was significantly increased in embryos exposed to concentrations exceeding 2.0μg/mL propofol, and increased apoptosis or an imbalance of apoptotic proteins may have also contributed to the delayed differentiation in embryos exposed to 2.0-4.5μg/mL propofol.

Although propofol has previously been shown to have a protective effect against mitochondrial dysfunction by inhibiting mitochondrial membrane permeability and reducing mitochondrial ROS production,[243] significant alteration of the redox-balance may still result in mitochondrial dysfunction and induce oxidative damage to cellular proteins.[198] Mitochondrial damage or alteration of membrane and intracellular proteins can perturb metabolism, which could affect nearly every process critical to early development. Propofol has the ability protect against mitochondrial dysfunction,[243] but excessive propofol exposure could potentially affect the intracellular redox balance to damage organelles, DNA, RNA, and proteins. Disruption of mitochondrial function would primarily affect metabolism during pre-compaction stages, when energy production is nearly entirely dependent on the mitochondrial tricarboxilic acid (TCA) cycle.[179,188,189] Oxidative stress has previously been found to cause cells to shift from oxidative use of pyruvate in the full TCA cycle to use of the segment of the TCA cycle involving succinate and succinate dehydrogenase.[198] Alteration of the TCA cycle would significantly interrupt the main mechanism of ATP production during early embryonic differentiation, possibly contributing to the significant degeneration and

2-cell block in embryos cultured at concentrations exceeding 2.5μg/mL propofol, as well as embryonal delay at concentrations between 2.5-4.5μg/mL propofol.

Embryonic dependence on the TCA cycle during the pre-compaction stages shifts to a metabolism based on glycolysis between the morula and blastocyst stages.[179,188,189] Between the 8-cell stage and morula, glucose consumption increases dramatically as embryo metabolism transitions to depend on glycolysis,[179,190] and by the blastocyst stage glucose is the primary energy substrate.[166] Glucose is not utilized as the main energy source prior to compaction,[166] but glucose uptake has been detected in earlier stages.[179] Though propofol inhibition glucose transport may not be directly involved the cleavage block at higher concentrations, it may have contributed to delayed differentiation. While the TCA cycle is dominant during early cleavages, the glucose transporter GLUT1 is expressed throughout development and is thought to provide the embryo with its basal glucose requirements.[193] Glucose uptake has previously been found to be interrupted by propofol,[48] and if propofol has similar effects in the embryo, deprivation of the primary energy substrate during later cleavage stages and compaction[166,179,190] would significantly affect many processes critical to development. Interference with facilitators of glucose uptake such as GLUT1 has been shown to result in a high rate of apoptosis at the blastocyst stage,[244] and inhibition of GLUT1 and associated decreased glucose transport previously found to be induced by propofol may have a similar effect. Inhibition of glucose transport may significantly slow cellular processes to significantly affect further cleavage, resulting in arrest prior to the blastocyst stage similar to that found to be elevated in groups exposed to concentrations exceeding 1.5μg/mL propofol. Increased apoptosis resulting from interference with facilitators of glucose uptake by propofol may have also contributed embryonal delay demonstrated by embryos cultured at 2.5, 3.0, 3.5μg/mL and 4.0μg/mL propofol; a significant percentage were halted at the morula stage, indicating that an exogenous factor inhibited differentiation to the blastocyst stage. Furthermore, at least 30% of embryos at concentrations between 3.5-4.5μg/mL propofol were arrested between 4-cells and 12-cells, a period of differentiation during which embryonic metabolism begins to transition from a reliance on the TCA cycle to glycolysis (Table 2).

The embryonic cell cycle can be modified either by introduction of new components, substrates, pathways, or altered interactions between them.[232] Inhibitory effects found in embryos arresting at the 2-cell stage suggest significant modification to the cell cycle occurred, especially at concentrations greater than 5.0μg/mL where 2-cell block was recorded in 100% of embryos. The cell cycle may have been affected by antimitotic activity exerted either directly or by propofol-induced oxidative stress. Later divisions may also be interrupted by exposure to exogenous factors.[232] Antimitotic effects resulting from propofol exposure could be exerted by a number of mechanisms, including destruction of the spindle and impaired chromatid separation during mitosis, or inactivation of Cdc25 phosphatases. Cell cycle control proteins Cdc25 phosphatases[235] may be inactivated by ROS, which has been found to lead to a block of the cell cycle progression.[236] Propofol may also have induced improper attachment of kinetochores to microtubules, which could trigger the spindle assembly checkpoint (SAC), and prevented the onset of

anaphase to arrest mitosis.[210] Several anesthetics (dibucaine, tetracaine, procaine, and lidocaine) have previously been found to significantly affect the organization of cytoskeletal components and plasma membrane attachment points[223] and similar effect may be produced by an anesthetic such as propofol.

The significant variation in sensitivity to propofol at the 3.0μg/mL and 3.5μg/mL concentrations (Table 2; Fig. 8) may be attributed in part to differences in sensitivity between embryos at various points within the cell cycle during initial exposure. During the G1 phase of the cell cycle, preimplantation embryos are particularly susceptible to environmental factors.[15] The timing of teratogen exposure during the cell cycle is consequently very important to embryonic survival. At first contact, affected embryos may have been at a transition in stages of the cell cycle, depending on the timing of insemination possibly contributing to the difference in sensitivity to propofol at the 4.5μg/mL concentration. At this concentration, 70.6% of embryos arrested and degenerated, and 50% degenerating embryos were able to complete at least one cellular division (Fig. 8), but a significant percentage still reached the morula and blastocyst stage.

Propofol exposure may have interfered with zygotic gene activation (ZGA), contributing to arrested or delayed embryonic differentiation. The failure to undergo ZGA often results in arrested progression,[170,177] similar to that observed in treatment groups exposed to concentrations greater than 2.0μg/mL propofol, where significant arrest of 2-cell embryos was observed. Direct mutagenic activity has not been found to result from propofol exposure, though. Previous tests of propofol have found neither direct mutagenic activity in the *Salmonella* mutation test, nor cause genetic mutation or gene conversion in *Saccharomices cerevisiae*.[43] Instead, oxidative stress exerted by propofol may be a more likely factor affecting ZGA in this study. The preimplantation embryo is particularly sensitive to oxidative stress during ZGA, and any condition which enhances ROS accumulation during ZGA can have detrimental consequences.[190] Under normal conditions, ROS increase has been found to culminate during ZGA, suggesting that during this transition, oxidant defense is depleted and must be replenished by ZGA.[190] Future experimentation is necessary to determine whether the higher doses of propofol (>2.0μg/mL) directly affected ZGA, or if oxidative damage to transcription was responsible for the developmental block at the 2-cell stage or altered the embryonic genome to delay differentiation.

Chromatin remodeling is necessary for the initiation of ZGA and the onset of embryonic transcription.[170] Chromatin remodeling uses energy from ATP hydrolysis to restructure or move nucleosome constraints to transcription to make the genome accessible to transcription factors, however defective transcription factor function can result in developmental arrest.[197,225,246] Interference with chromatin remodeling or transcription factors may have contributed to the inhibition observed in the embryos exposed to greater than 2.0μg/mL propofol. Culture media has previously been found to directly affect gene expression (actin, G-3PDH, and Na^+/K^+ATPase housekeeping genes), transcription factors, and growth factors, as well as impacting the rate of degradation of mRNAs which can affect methylation and expression,[228] but further study of propofol effects is necessary.

The results of this study suggest that exposure to propofol affects preimplantation murine embryo development by delaying or arresting cleavage. At propofol concentrations well below the clinical ED, in vitro preimplantation embryonic progression to blastocyst was inhibited; all concentrations exceeding 1.5μg/mL propofol significantly delayed or arrested differentiation. Further testing concentrations within the range of embryonal delay (2.0-5.0μg/mL propofol) should enable detailed analysis of potential molecular mechanisms contributing to the inhibition of preimplantation embryonic development by propofol. Detailed examination of propofol effects on preimplantation embryo biochemistry is necessary to confirm that apoptosis is indeed activated by propofol exposure, that increased apoptosis contributed to inhibited differentiation, and to elucidate the mechanism(s) by which apoptosis is initiated. Additional mechanistic study is needed for characterization of the significant 2-cell block recorded at propofol doses exceeding 1.5μg/mL, and may find a correlation between either direct mutagenic effects or propofol-exerted oxidative stress effects resulting in arrested cleavage. Continued biochemical investigation of propofol effects in the preimplantation embryo should also provide insight into the mechanism by which inhibition is exerted. DNA and RNA damage, interference with transcription and transcription factors, suppression of HSP expression, antimitotic effects and spindle formation, mitochondrial interactions, initiation of the apoptotic cascade, interruption of metabolism and nutrient uptake, or a combination of any of these factors should allow a greater understanding of the inhibition caused by propofol. Many of these factors may be investigated by experimentation in non-embryonic cells such as fibroblasts, mouse embryonic stem cells, mammalian micromass cell culture, or sperm cell viability, which would allow decreased expenses and animal use for abundant and timely data collection prior to testing effects in the preimplantation embryo. Future study of embryo transfer for implantation and fetal development may also provide evidence to support significant effects of propofol on embryonic development does indeed exist and may potentially result in a decrease in live pup births (embryo or fetal resorption) or an increase in teratogenesis/birth defects.

CHAPTER 3

SODIUM PENTOBARBITAL

INTRODUCTION

The highest developmental sensitivity to barbiturates has been found during early stages of chick embryo development at subclinical levels, a period which may also be clinically relevant in mammals.[81] Short and long-term consequences of prenatal and neonatal exposure to barbiturates in humans include changes in the CNS and behavior;[85] and congenital malformations associated with phenobarbital exposure include midface and digit hypoplasia,[219] and microcephaly.[216,219] The FDA classifies pentobarbital to be a class D potentially teratogenic medication,[1] however little published research supports the potential of pentobarbital to be detrimental to development.[14,81,84,252,253] Pentobarbital exposure during neural development has been found to significantly affect neurological physiology and behavioral function.[84] GABAmimetics such as barbiturates are capable of triggering neuroapoptosis in the developing brain.[116] Pentobarbital has the potential to destroy newly formed neurons resulting in behavioral and cognitive defects; and even early administration of the less potent barbiturate phenobarbital has been found to impair the rat hippocampus and cause extensive deficiencies in radial maze behavior and spontaneous alteration.[85] Prenatal exposure to barbiturates has been found to cause syndromes of neurobehavioral deficits, especially involving defects in septohippocampal cholinergic innervation-related behaviors.[215] Eight-arm radial maze performance is the most common test of impairment to the septohippocampal pathways,[217] and extensive deficiencies in radial maze performance and spontaneous alteration behaviors have been found to be *associated with barbiturate administration during neural development.*[85]

Documentation of the effects of barbiturates on preimplantation embryo development is sparse,[14] and extensive search of the literature has revealed an absence of in vitro studies of pentobarbital effects in the preimplantation embryo. Most studies of developmental effects of barbiturates focus on late-term exposure and are limited to teratological examination for visual determination of major malformations in the offspring, and behavioral studies examining effects on neurological development. Effects of sodium pentobarbital (NaPB) anesthesia on the preimplantation embryo have been investigated in rabbits, though, where female rabbits were injected intravenously at either 15 minutes or 6h after mating.[14] Blastocysts were recovered from females at gestation day 6 and examined for chromosomal abnormalities; a greater frequency of chromosomally abnormal blastocysts was found in animals injected with pentobarbital than controls.[14]

The anesthetic use of barbiturates has diminished in recent years,[15,23,30,33-43] however the long history of barbiturate use and research, as well as utilization for selective procedures or conditions, and potential for abuse compels continued barbiturate research. NaPB anesthesia remains prevalent in many undeveloped

countries,[82] and NaPB use continues as a second or third-line agent in first-world countries for a number of applications.[24,73,82,84-87] The decades of use of NaPB, in addition to its generalized availability, low cost, relatively rapid onset of anesthesia, and ease of injection to a variety of animals contributes to its sustained widespread use for laboratory animals and for veterinary medicine.[68,82,85,88] Few IV barbiturates are currently used, however NaPB and thiopental are common agents, and the availability issues of thiopental[82] influenced the selection of NaPB as a representative of barbiturates for this study.

The focus of this study was to evaluate the preimplantation embryotoxicity of NaPB. Toxicity of long-term exposure to NaPB in the 2-cell preimplantation embryo is previously unreported. The mouse system is valuable for screening the potential toxic effects of any xenobiotic compound which may come in contact with a developing embryo,[138] and the mouse embryo growth and development 72h bioassay is considered to be by the clinical reproductive industry the preferred assay for quality assurance/quality control (QA/QC) determination and validation of gamete and embryo research.[153] The window during preimplantation development, and the dosage and severity of embryotoxic effects which may be exerted by NaPB has not been previously evaluated. Early embryonic stages provide indications of potential induction of fetal and postnatal defects,[157] and extended exposure allows determination of effects of later preimplantation development since some components have the ability to affect the development of the blastocyst and inner cell mass directly.[151] Evaluation of extended drug exposure is necessary since some components causing failure of the bioassay may compromise embryo development without being outright lethal.[151] Extended exposure may also assist in evaluation of potential embryotoxicity of pharmaceutical agents subject prolonged use or habitual abuse.

This study was performed with the objective of evaluating the potential of NaPB to impair cleavage through the normal progression of in vitro embryonic development from the 2-cell to the blastocyst stage, and the developmental timing and extent to which damage may be exerted. Potential adverse effects of NaPB were assessed by individual culture of preimplantation 2-cell murine embryos with incremental concentrations of NaPB, starting with normal clinical concentrations and surpassing the normal clinical time of exposure. When extreme embryotoxicity was observed near the range of clinical concentrations for rodents (300μg/mL)[93-96] during preliminary exploratory experimentation, the dosages of NaPB were modified to a serial dilution of normal clinical anesthetic dose (30μg/mL, 3.0μg/mL, and 0.3μg/mL) to evaluate embryotoxicity in follow-up experimentation. Embryotoxicity was assessed by analysis of early cleavage and subsequent differentiation of 2-cell preimplantation embryos subjected in vitro to prolonged 72h exposure of NaPB. Detection of abnormal embryo morphology including fragmentation, granulation, and degeneration of blastomeres provided indications of deleterious clastogenic damage capable of significantly inhibiting in vitro development.

METHODS

Animals

Embryos used in the study were produced on IACUC protocol #12-017. Embryos were obtained from female mice 6-18 weeks old (B_6CBAF_1F, purchased from Jackson Laboratories, Bar Harbor, Maine) crossed with outbred CD-1 males 2-10 months old (Harlan Lab, Indianapolis, IN). This F2 hybrid mouse strain has proved to produce consistently high numbers of embryos with 100% development to blastula stage in 72h if all culturing variables are at an optimum. Mice were 4-6 weeks old when received and acclimated to the animal facility for at least 48h before use and were used within 12 weeks of purchase. Mice were maintained on a lighting regime of 14h light, 10h dark at 21°-25°C and given water and laboratory rodent chow (Harlan) *ad libitum*. Euthanasia was performed by cervical dislocation, approved by the Panel on Euthanasia of the American Veterinary Medical Association.

Superovulation And Mating

Female mice were selected at random for exogenously driven superovulation. Superovulation was achieved by intraperitoneal (IP) injection of gonadotropic hormones. IP injection was performed through the lower right quadrant of the abdomen using a 1mL syringe with a ½ inch 30 gauge needle. Beginning four days prior to use, the injection cycle consisted of two injections separated by 48h. The first injection at 0h was 0.1mL of a 50 international units (IU)/mL (5 IU) of pregnant mare serum gonadotropin (PMSG) (SIGMA, G4877, 106K1171; St. Louis) in phosphate buffered saline (PBS). At 48h after PMSG injection, 0.1mL of a 50 IU/mL (5 IU) solution of human chorionic gonadotropin (hCG) (SIGMA, CG5, 075K1442; St. Louis) in PBS was administered. At the time of hCG injection, each female was caged with one proven fertile male CD-1 mouse. Female mice go into estrous and mate within 6h of hCG injection, with ovulation occurring between 10 and 12h after hCG injection. Between 8 AM and 10 AM (16h after hCG injection) the following morning, female mice were inspected for vaginal copulatory plugs and the result of the performance of each male was recorded. At this time mated and unmated females were segregated and marked appropriately with cage cards; only mated animals were used. Unmated animals were submitted to the same injection cycle after a two-week recovery period during which the pituitary gland is less sensitive to gonadotropins. Females cycled three times without successful mating were removed and euthanized by cervical dislocation.

Collection Of Embryos

Females with vaginal copulatory plugs were euthanized using cervical dislocation starting approximately 42h after the hCG injection (pregnancy day 0) and immediately prior to embryo collection. The abdomen of the mouse was entered by producing a small hole above the umbilicus then retracting the skin simultaneously down over the hind legs and over the forelegs in a process known as de-gloving. This technique leaves a sterile field for entry without any mouse hair and obviates use of antiseptic agents that

may compromise embryo viability. Bilateral celiotomy was performed to expose the oviducts of pregnant animals. Salpingectomy was performed by gently stretching and tearing the mesosalpinx and mesometrium away from each oviduct and uterine horn, and then cutting between the oviduct and ovary at the cephalad end and the oviduct and the intramural junction at the caudal end with microsurgical scissors. The two oviducts were then placed in each sterile 35 x 10mm polystyrene culture dish (Nalge Nunc International) containing 2mL of modified Krebs-Ringer-bicarbonate (mKRB) medium (See Appendix 1). A Zeiss dissecting stereomicroscope (Eastern Microscope Co, Raleigh, N.C.) at 20x magnification, watchmaker forceps (Dumont and Fils, Switzerland) was used to slide the fimbriated end of the oviduct onto a 30-gauge (FineJect, Henke Sass Wolf) sterile needle attached to a 1 mL sterile syringe (Norm-Ject). Embryos were irrigated from the oviducts with medium lightly expressed from the syringe. Each animal can be expected to produce an average of 15 embryos per successful mating (successful mating is approximately 1 per 2 stimulated females = (½)).

Embryos

Individual morphologically normal (judged by oval, bilateral symmetry of the two blastomeres with narrow perivitelline space and presence of two small polar bodies) 2-cell embryos was evaluated at 32x magnification and collected from the petri dish with a 10µL micropipette (Eppendorf) fitted with a sterile tip (Avant) and aseptically dispensed into 96 well U-bottom culture plates (Falcon, 35-3077) with one embryo per well. Embryos were cultured in a NuAire Autoflow IR Water Jacketed CO_2 Incubator at 37°C, 5% CO_2 for 72h. Differentiation was evaluated every 24h at 40x and 100x using a Nikon Diaphot inverted microscope. Each test medium was evaluated in triplicate cultures.

Solutions And Media

Media used was mKRB solution supplemented with 4mg bovine serum albumin (BSA) per mL (See Appendix 1) and conditioned at 37°C 18-24h. Sodium pentobarbital (NaPB) (60mg/mL) (Sigma, P3751) buffered with 20% glycerol 10% ethyl alcohol to improve miscibility was dissolved in mKRB media at designated concentrations and equilibrated for at least 12h before use. The dosage of NaPB was adjusted to 10^{-1}, 10^{-2}, and 10^{-3} relevant anesthetic dose (300µg/mL)[93-96] following the extreme toxicity observed at 300µg/mL in preliminary experimentation. The mKRB media containing the addition of 20% glycerol 10% ethyl alcohol was used as the negative control for the pentobarbital experiments, since the initial pentobarbital positive control of mKRB media containing 20% glycerol 10% ethyl alcohol produced no significant effect.

Statistics

A 72 hour embryo culture endpoint with minimum development of 65% blastula and combined 80% morula + blastula embryo morphology is necessary to report a passing score on the mouse embryo assay. Cell number and overall developmental stage of embryos in specific treatments were analyzed using

one-factor fixed-effects randomized block analysis of variance (ANOVA) performed using IBM SPSS 20 to test for overall significance among treatments. If significance was found, this was followed by Tukey's HSD (honestly significant difference) test (for unequal sample sizes in treatment groups) for differences between means of specific treatments. The Shapiro-Wilk test of normality and Levene's test of equality of variance were also used to evaluate whether samples were normally distributed and if there was statistically significant variation between treatments. The significant groups were then tested with χ^2 for homogeneity and difference of proportions. Culture of at least 15 embryos is performed with each item that we test to meet the CLIA '88 federal law to establish toxicity for the tested item. This embryo number (15) was established by the R.J. Swanson Mouse Embryo Laboratory as the standard for their testing protocol.

RESULTS

Results of NaPB experiments are presented in Table 3 and Table 4. Significant inhibitory effects to mouse embryo differentiation were found at 300μg/mL and 30μg/mL pentobarbital concentrations (Table 4). Preliminary experimentation of the rodent anesthetic dose (300μg/mL)[93-96] found NaPB to be 100% toxic to 2-cell mouse embryos, with 100% cleavage arrest of 2-cell embryos characteristic of the "2-cell block." NaPB dosage was modified to 10^{-1}, 10^{-2}, and 10^{-3} ED for follow-up experimentation. Positive control experiments of the vehicle media (20% glycerol 10% ethyl alcohol with mKRB media containing BSA) were performed alongside mKRB media. Preliminary study of the vehicle produced a passing embryo score, with 100% of embryos reaching blastocyst when cultured in the positive control solution. Vehicle media was used as the negative control for follow-up experimentation.

Culture of embryos with NaPB significantly decreased differentiation to the blastocyst stage at 30μg/mL; however all lower concentrations did not significantly affect cleavage and differentiation to the blastocyst stage. Significant degeneration and visible nuclear fragmentation in 53.8% of embryos treated within the 30μg/mL NaPB group ($\chi 2$, $p<0.001$) (Table 4) was observed. Only 30.7% of embryos within the degenerating/fragmenting group completed the second cleavage division by the 72h endpoint. In addition, at the 72h endpoint, 23.1% of embryos at 30μg/mL NaPB reached the compact morula stage, and only 23.1% of embryos at this concentration reached the blastocyst stage. Effects of NaPB on blastocyst development were also tested with χ^2 for homogeneity and difference of proportions within significant groups. Inhibition of blastocyst hatching compared with blastocyst formation was significant at 0.3μg/mL ($\chi 2$, $p<0.01$), 3.0μg/mL ($\chi 2$, $p<0.001$), and 30μg/mL NaPB (0% hatching) (Table 4).

Results of the one-factor fixed-effects randomized block ANOVA for overall significance among treatments found a significant effect between development groups (degenerating/ fragmenting, morula, and blastocyst) ($F=8.494253$; $df=2,9$; $P=0.008464$), and a significant difference between treatment concentrations ($F=6.013$; $df=3,8$; $P=0.041$). This probability is less than the alpha value of 0.05 associated with a 95% confidence level. Significant effects were present at the 30μg/mL concentration, however all lower concentrations were found to have no significant effects on differentiation. This analysis was

followed by Post Hoc Tukey's HSD test to evaluate differences between specific treatments. The Tukey's HSD test did not reveal significant effect between specific blocks. The Shapiro-Wilk test of normality indicated that the samples were not normally distributed (P=0.034, 0.062, 0.024). Levene's test of equality of variance fell outside of the critical realm (F=2.499; d*f*=3,8; P=0.134), indicating that NaPB did not have a significant effect on differentiation at concentrations below 30µg/mL.

Table 3. Sodium pentobarbital 72h effects on embryonic development: percentage of embryos reaching specific stage of differentiation.

NaPB µg/mL	2-Cell	4-Cell-12-Cell	Total Degenerating	Morula	Blastocyst	Total Cultured
0 (Control)	3.4%	0%	3.4%	3.4%	93.1%	29
0.3	0%	0%	0%	0%	100%	27
3.0	3.8%	0%	3.8%	3.8%	92.3%	26
30	23.1%	30.7%	53.8%	23.1%	23.1%	26
300	100%	0%	100%	0%	0%	10

Differentiation results of NaPB buffered with mKRB media+BSA. Degenerating refers to abnormal development or deterioration of the embryo before the compact morula stage. A control was performed using mKRB+BSA embryo culture media sans NaPB; however the NaPB used in these experiments was dissolved in 10% ethyl alcohol, 20% glycerol. Preliminary experimentation comparing effects of the mKRB+BSA embryo culture media with mKRB+BSA+vehicle (10%EtOH+20%glycerol) did not produce significant results (both yielded passing embryo scores). The mKRB+BSA+vehicle solution was used as control for follow up experiments.

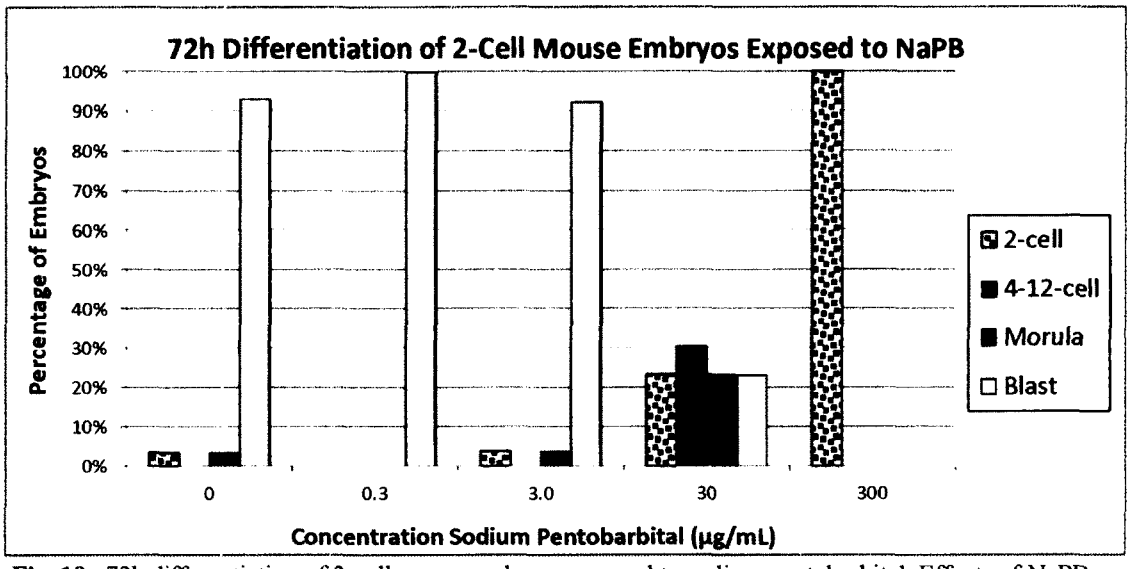

Fig. 10. 72h differentiation of 2-cell mouse embryos exposed to sodium pentobarbital. Effects of NaPB indicated by percentage reaching each embryonic stage of differentiation. NaPB was buffered with mKRB media+BSA. Control solution contained mKRB+BSA+vehicle (10% EtOH+20% glycerol) sans NaPB. Blast refers to blastocyst (32+ cells).

Table 4. Sodium pentobarbital 72h effects on embryonic development: detailed differentiation percentages.

NaPB µg/mL	2-Cell	4-Cell	8-Cell-12-Cell	Total Degenerating	Morula	Early Blast	Hatching Blast
0 (Control)	3.4%	0%	0%	3.4%	3.4%	51.7%	41.4%
0.3	0%	0%	0%	0%	0%	33.3%	66.7%
3.0	3.8%	0%	0%	3.80%	3.8%	65.4%	27%
30	23.1%	15.35%	15.35%	53.8%	23.1%	23.1%	0%
300	100%	0%	0%	100%	0%	0%	0%

NaPB buffered with mKRB media+BSA was tested. Degenerating refers to abnormal development or deterioration of the embryo before the compact morula stage. Control solution contained mKRB+BSA+vehicle (10%EtOH+20%glycerol) sans NaPB. Blast refers to blastocyst (32+ cells).

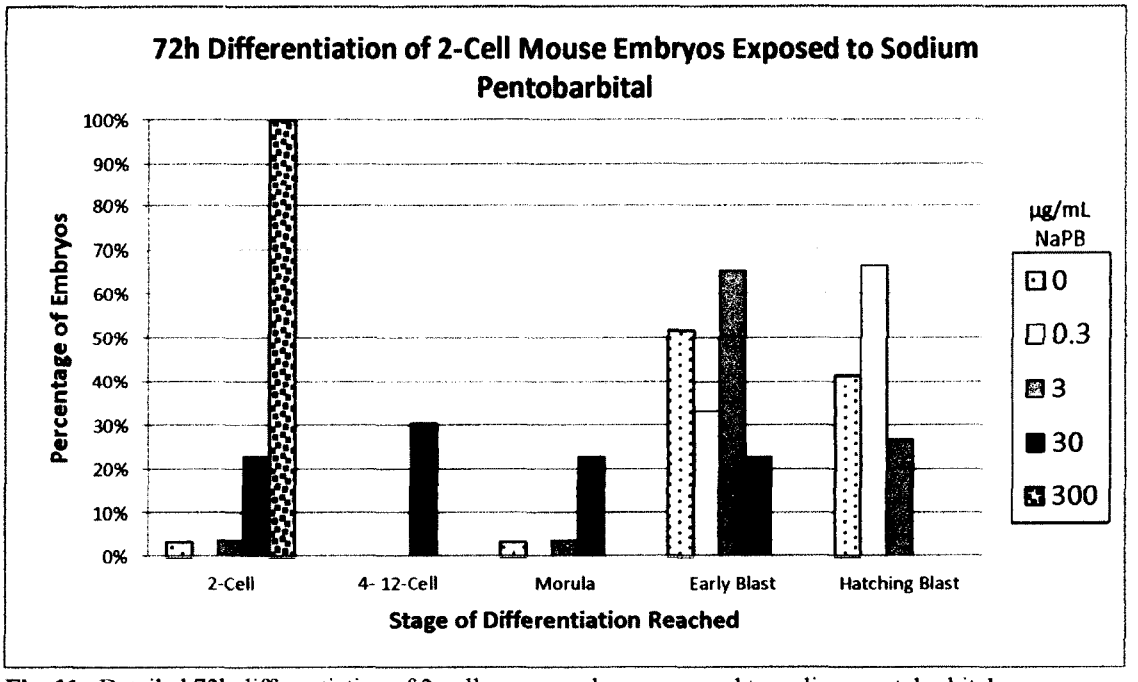

Fig. 11. Detailed 72h differentiation of 2-cell mouse embryos exposed to sodium pentobarbital. Differentiation effects indicated by percentage of embryos reaching specific stage of differentiation. NaPB was buffered with mKRB media+BSA. Control solution contained mKRB+BSA+vehicle (10% EtOH+20% glycerol) sans NaPB. Blast refers to blastocyst (32+ cells).

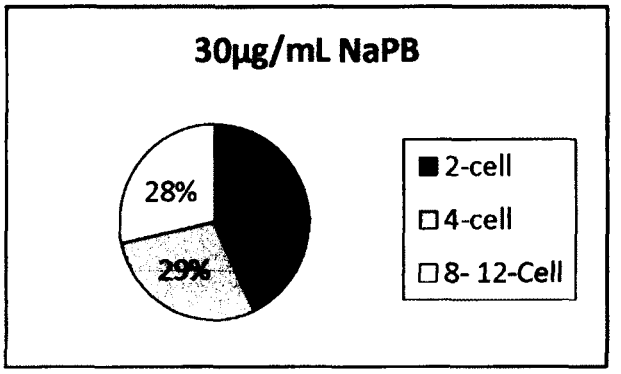

Fig. 12. Differentiation progress of degenerating embryos following 72h exposure to 30µg/mL sodium pentobarbital. Percentages of embryos arrested at the 2-cell stage in comparison to embryos able to undergo at least one cleavage division before degenerating and/or fragmenting (4-cell and 8- 12-cell). Significance between stages of differentiation of degenerating embryos was only found at this concentration.

DISCUSSION

Results of this study suggest that the presence of NaPB produces dose-dependent inhibitory effects on in vitro cultured 2-cell mouse embryos. Cleavage and differentiation to the blastocyst stage of preimplantation mouse embryos at the 2-cell stage was impeded when exposed to extended culture at 30µg/mL and 300µg/mL NaPB. At these concentrations, significant embryotoxicity was observed within the first 48h of culture and affected the first cleavages. The decreased progression of 2-cell embryos to blastocysts during 72h culture indicates embryonal arrest during the first cleavages (two- to eight-cell stage), and 100% blastomere degeneration and fragmentation during exposure to the highest dose is suggestive of extreme embryotoxicity. Differentiation was completely inhibited in 100% of 2-cell embryos exposed to the highest concentration tested, 300µg/mL NaPB, where fragmentation and degeneration was present in all embryos cultured at this concentration. Although the industry standard for establishing embryotoxicity requires culture of at least 15 embryos per treatment, the 100% toxicity found in 10 embryos during preliminary investigation of embryotoxicity of the relevant clinical ED (300µg/mL) NaPB was sufficient indication of a necessity to decrease concentration for additional study. This assumption was supported by the wide range of differentiation of embryos observed at the next lowest treatment (30µg/mL) once dosages were modified (Table 4; Fig.11).

At 30µg/mL NaPB, a significant percentage of embryos displayed a 2-cell block where no cleavage divisions occurred. A significant amount of embryos were, however, able to complete one or several cleavage divisions before arresting at this concentration (Fig. 11; Fig. 12), as well as a significant percentage able to reach the compact morula and early blastocyst stage. Though a significant percentage of embryos were able to reach later stages of development at 30µg/mL NaPB, the combined morula + blastula embryo morphology score of 46.2% fell well outside the necessary passing score of 75% for human IVF,[144] and 80% for the mouse embryo bioassay.[251] At 0µg/mL and 3.0µg/mL (relevant human

anesthetic ED),[24,73] the embryos degenerating at the 2-cell stage (less than 4%) are not indicative of a significant effect. It is not uncommon for a small percentage of embryos to fragment or degenerate even when cultured with specific embryo culture media.[144]

Statistical analysis was performed for embryo morphology groups commonly used to evaluate toxicity in the 72h mouse embryo bioassay, degenerating, morula, and blastocyst. Detailed examination of differentiation, however, produced compelling results when analyzed using χ^2 for homogeneity and difference of proportions. The degeneration and nuclear fragmentation in 53.8% of embryos treated at 30μg/mL NaPB was significant (Table 4). An apparent significant effect between embryos degenerating at 30μg/mL, where 43% displayed a 2-cell block, 29% completed one cleavage division to arrest at the 4-cell stage, and the remaining 28% degenerated prior to reaching the morula stage falls outside the critical realm for significance ($\chi 2$, $p=0.1633$). Significant effects were found between those degenerating, and those reaching the compact morula stage (23.1%) or the early blastocyst stage (23.1%) ($\chi 2$, $p<0.001$). This effect was statistically significant and is indicative of inhibition in response to embryonic exposure to 30μg/mL NaPB. The difference between the groups treated with 3.0μg/mL and 0.3μg/mL NaPB, and control (mKRB+BSA+EtOH+Glycerol), was not found to be statistically significant. This study found insufficient evidence to support the assumption that the lower doses (<30μg/mL) of NaPB tested have a significant inhibitory effect on the ability of murine embryos to reach the blastocyst stage, however analysis of later cleavage stages reveals a fascinating trend. As the concentration of NaPB increases from 0.3μg/mL to 30μg/mL, the ratio of embryos reaching the hatching blastocyst stage to the early blastocyst stage significantly decreases (0.3μg/mL $\chi 2$, $p<0.01$; 3.0μg/mL $\chi 2$, $p<0.001$). This effect is likely a result of NaPB interference with later embryonic processes involved with blastocyst development and maturation and will be discussed below.

The embryotoxicity of NaPB exposure in the current study may be attributed to a variety of biochemical mechanisms or a combination of deleterious effects. Exogenous compounds can affect development of the preimplantation embryo by increasing oxidative stress, and due to relatively weak antioxidant defense during early developmental stages,[227] this stress often results in defective development.[224] Oxidative stress, which has previously been found to produce a developmental block in mouse embryos cultured from the 2-cell stage,[224] is a likely factor impeding differentiation in embryos cultured in the higher concentrations of NaPB in this study. Although pentobarbital has previously been found to suppress free radical generation and increase antioxidant ability,[239] NaPB has also been found to aggravate oxidative stress damage in some tissues.[240]

The ability of a significant percentage of embryos to complete at least one cellular division before degenerating at 30μg/mL, suggests that at this concentration, many embryos were able to endure toxic effects long enough to stagger through at least one additional cleavage. This survival effect may be attributed to moderate toxicity at 30μg/mL NaPB only affecting select cellular processes, perhaps in combination with the capacity of embryonic endogenous mechanisms to preserve some normal functions in

the presence of environmental insult. Embryonic repair may also have assisted in survival at this concentration; undifferentiated cells of the early embryo can make repair possible through multiplication of cells,[4] even if development may still be compromised by non-lethal insults.[151,224]

Induced expression of HSPs due to suboptimal culture conditions and constitutive HSP expression both represent an essential mechanism for successful embryo growth in an adverse environment, however inducible expression is not active until the morula or early blastocyst stage.[178] Inadequate HSP expression at the time of initial NaPB contact may have contributed to inability of 2-cell embryos to recover from the chemical insult of exposure at 30μg/mL and 300μg/mL NaPB. Loss of heat shock factor function has previously been found to result in developmental arrest as early as the 2-cell stage[197,225,246] similar to the 2-cell block at 30μg/mL and 300μg/mL NaPB recorded in this study. Early HSP expression may have also assisted in the survival and additional cleavage divisions of a significant percentage of embryos at 30μg/mL if this concentration did not overwhelm the minor protective effects of early HSP expression. Inducible HSP expression may have contributed to the trend of delayed later development of the embryos at 0.3, 3.0μg/mL, and 30μg/mL NaPB as well. This suggests that NaPB exposure affected later processes involved with formation and maturation of the blastocyst at 0.3μg/mL and 3.0μg/mL without interfering with early cleavage divisions. The induced expression of HSPs up regulated during the morula to blastocyst stage may have significantly assisted resistance to NaPB to allow later cleavage and blastocyst formation. Although inhibition of early cleavage up to the morula stage was insignificant at 0.3μg/mL and 3.0μg/mL, as NaPB concentration increments increased between 0.3μg/mL and 30μg/mL, later differentiation was significantly affected (Table 4, Fig.11). The significant percentage of embryos delayed at the morula stage or only reaching the early blastocyst stage at 30μg/mL also supports this assumption.

Cellular fragmentation characteristic of apoptosis was significant in embryos exposed to 30μg/mL and 300μg/mL NaPB, and increased apoptosis or an imbalance of apoptotic proteins may have also contributed to delayed differentiation observed at 30μg/mL NaPB. In vitro induction of apoptosis may be attributed to suboptimal culture conditions, excessive ROS exposure, or chromosomal abnormalities,[178] and high levels of apoptosis have previously been detected in fragmenting preimplantation embryos.[238] The fragmentation of blastomeres observed in this study in degenerating embryos at 30μg/mL and 300μg/mL NaPB was indicative of apoptosis, however the exact mechanism by which the apoptotic cascade was initiated is unclear. Multiple stimuli such as oxidant insults, pro-apoptotic BAX protein activation, intracellular Ca^{2+} overload, and direct activation of other caspases are capable of activating caspase enzymes to initiate apoptosis.[173]

NaPB has previously been shown to increase oxidative stress,[240] which is capable of damaging mitochondrial membranes,[198] the primary mechanism by which initiation of apoptosis occurs.[173] Mitochondrial damage or alteration of membrane and intracellular proteins can perturb metabolism, which could affect nearly every process critical to early development. Mitochondrial function is critical for the progression of early development,[190] and in addition to initiation of apoptosis, disruption of mitochondrial

function would primarily affect metabolism during pre-compaction stages, when energy production is nearly entirely dependent on the mitochondrial TCA cycle.[179,188,189] Alteration of the TCA cycle would significantly interrupt the main mechanism of ATP production during early embryonic differentiation, potentially contributing to the significant degeneration and 2-cell block in embryos cultured at 30μg/mL and 300μg/mL NaPB. The delayed differentiation and arrest of cleavage prior to reaching the blastocyst observed in embryos at 30μg/mL NaPB may also be attributed to inhibition of early metabolism by NaPB-induced mitochondrial damage. Mitochondria are responsible for providing the energy required to maintain cellular activity through oxidative phosphorylation and provision of intermediates for glycolysis, as well as affecting ion homeostasis, participating in amino acid metabolism, signal transduction, and apoptosis.[196] In addition, ATP is necessary for embryonic compaction between the 8-cell and morula stage,[173] as well as for active transport to internalize fluid for formation of the blastocoel[165,169,171] to generate the blastocyst.[165] The expansion of the blastocoel is crucial to the differentiation of the ICM and "hatching" of the embryo from the zona pellucida to prepare for uterine implantation.[152,165,174] Later delayed differentiation observed in embryos at 0.3μg/mL, 3.0μg/mL NaPB may also be attributed to defective mitochondria metabolic effects, depriving embryos of necessary ATP for mitotic divisions and blastocyst differentiation; however NaPB may also have affected the trophectoderm Na^+/K^+ pumps necessary for blastocoel expansion.[173]

Mitochondrial dysfunction may not be the only mechanism by which NaPB potentially interrupted metabolism to cause embryonic delay of later differentiation. Embryonic dependence on the TCA cycle prior to compaction shifts to a metabolism based on glycolysis between the morula and blastocyst stages.[179,188,189] During the 8-cell to morula stage, glucose consumption increases dramatically and the embryo shifts its metabolism to depend on glycolysis,[179,190] and by the blastocyst stage, glucose is utilized as the primary energy substrate.[166] The glucose transporter GLUT1 is expressed throughout development and is thought to provide the embryo with its basal glucose requirements.[193] Interference with facilitators of glucose uptake such as GLUT1 has been shown to result in a high rate of apoptosis at the blastocyst stage of murine embryos.[244] This was attributed to hyperglycemia associated decreased glucose transport, acting as a cell death signal to trigger a BAX-dependent apoptotic cascade.[244] This correlation between hyperglycemia and elevated apoptosis during the blastocyst stage was proposed to result key progenitor cell loss, potentially manifesting as resorption, malformation, or miscarriage,[244] pregnancy outcomes increased in diabetic women.[244,245]

Pentobarbital has previously been found to interact directly with GLUT1 and to inhibit glucose transport.[48] Inhibition of glucose uptake previously found to be induced by pentobarbital in other tissues may also have similar apoptotic effects in the preimplantation embryo. This inhibition of glucose uptake by NaPB may have also interfered with the embryonic metabolic transition to glycolysis resulting in significant embryonic arrest between the 8-cell and morula stages at 30μg/mL NaPB (15.35%), as well as the significant percentage of embryos halted at the morula stage (23.1%) prior to blastocoel formation.

Down regulation of glucose transport may significantly contribute to the trend of embryonic delay of later differentiation between morula and blastula stages as NaPB dosage increments increased from 0.3-30μg/mL. As embryonic metabolism becomes dependent on glycolysis, inhibition of glucose transport may directly initiate apoptosis or significantly deprive the embryo of ATP necessary for differentiation and blastocoel development to slow or arrest progression.

The embryonic cell cycle can be modified either by introduction of new components, substrates, pathways, or altered interactions between them.[232] Inhibitory effects found in delayed embryos at 30μg/mL NaPB and arresting embryos at 30μg/mL and 300μg/mL NaPB suggest significant modification to the cell cycle occurred. Antimitotic effects resulting from NaPB exposure to induce the 2-cell block in embryos at 30μg/mL and 300μg/mL NaPB could be exerted by a number of mechanisms. Cell cycle control proteins Cdc25 phosphatases[235] may be inactivated by ROS, which has been found to lead to a block of the cell cycle progression.[236] NaPB may also have induced improper attachment of kinetochores to microtubules, which would trigger the spindle assembly checkpoint (SAC).[210] This effect would have prevented the onset of anaphase[210] and arrested the cell cycle. At 30μg/mL and 300μg/mL NaPB, the SAC likely contributed to the arrest and subsequent degeneration of 2-cell embryos. Several local anesthetics (dibucaine, tetracaine, procaine, and lidocaine) have previously been found to significantly affect the organization of cytoskeletal components and plasma membrane attachment points[223] and similar effect may be produced by a general anesthetic such as NaPB.

Oxidative stress is capable of directly causing DNA or RNA damage,[190,227] which may interfere with zygotic gene activation (ZGA). The preimplantation embryo is particularly sensitive to oxidative stress during ZGA, and any condition which enhances ROS accumulation during ZGA can have detrimental consequences.[190] Failure to undergo ZGA often results in arrested progression,[170,177] comparable to the 2-cell block of embryos exposed to 30μg/mL and 300μg/mL NaPB. Chromatin remodeling is a necessary process for successful ZGA and embryonic differentiation.[170] Chromatin remodeling uses energy from ATP hydrolysis to restructure or move nucleosome constraints to transcription to make the genome accessible to transcription factors, however defective transcription factor function can result in developmental arrest.[197,225,246] Culture media has previously been found to directly affect gene expression (actin, G-3PDH, and Na^+/K^+ATPase housekeeping genes), transcription factors, and growth factors, as well as impacting the rate of degradation of mRNAs which can affect methylation and expression.[228] Interference with chromatin remodeling or transcription factors may have been another contributor to the 2-cell block of embryos exposed to 30μg/mL and 300μg/mL NaPB. The zygote and the 2-cell embryo are also susceptible to certain mutagens with a delayed expression of damage,[157] which may have been another factor contributing to the embryonal delay in this study. Future experimentation is necessary to evaluate NaPB mutational effects of maternal-effect genes and ZGA, or oxidative stress exerted by any of these concentrations affected ZGA to delay differentiation or cause a developmental block at the 2-cell stage.

The results of this study suggest that subclinical concentrations of NaPB affect preimplantation mouse embryo development by delaying or arresting early cleavage. NaPB at 30μg/mL and 300μg/mL significantly inhibited embryonic differentiation to the blastocyst stage. Lower concentrations 0.3μg/mL and 3.0μg/mL NaPB did not significantly inhibit blastocyst formation; however 3.0μg/mL exerted significant inhibitory effects on later differentiation including blastocyst maturation and hatching compared to controls and 0.3μg/mL NaPB. This should assist recommendations regarding the use of NaPB anesthesia during early pregnancy in humans since these later preimplantation inhibitory effects were observed at a concentration within the range of the relevant human ED (2-4mg/kg, 2.0-4.0μg/mL).[24,73] Further testing of concentrations between 0.3μg/mL and 300μg/mL NaPB should provide insight into the range of embryonic effects within the range relevant to veterinary/laboratory anesthesia and enable detailed analysis of potential molecular mechanisms contributing to the inhibition of preimplantation embryonic development by NaPB.

Detailed examination of NaPB effects on preimplantation embryo biochemistry is necessary to confirm that apoptosis is indeed activated by NaPB exposure, that increased apoptosis contributed to inhibited differentiation, and to elucidate the mechanism(s) by which apoptosis is initiated. Additional mechanistic study is needed for characterization of the significant 2-cell block recorded at NaPB doses exceeding 3.0μg/mL, and may find a correlation between either direct mutagenic effects or NaPB-exerted oxidative stress effects resulting in arrested cleavage. Continued biochemical investigation of NaPB effects in the preimplantation embryo should also provide insight into the mechanism by which inhibition is exerted. DNA and RNA damage, interference with transcription and transcription factors, suppression of HSP expression, antimitotic effects and spindle formation, mitochondrial interactions, initiation of the apoptotic cascade, interruption of metabolism and nutrient uptake, or a combination of any of these factors should allow a greater understanding of the inhibition caused by NaPB. Investigation of later differentiation may also provide insight into how either increased apoptosis or ATP deprivation resulting in insufficient energy to sustain further mitosis or power Na^+/K^+ pumps necessary for blastocoel development may affect the inhibition of blastocyst hatching in progressively increasing NaPB concentrations. Many of these factors may be investigated by experimentation in non-embryonic cells such as fibroblasts, mouse embryonic stem cells, mammalian micromass cell culture, or sperm cell viability, which would allow decreased expenses and animal use for abundant and timely data collection prior to testing effects in the preimplantation embryo. Future study of embryo transfer for implantation and fetal development may also provide evidence to support significant effects of NaPB on embryonic development does indeed exist and may potentially result in a decrease in live pup births (embryo or fetal resorption) or an increase in teratogenesis/birth defects.

CHAPTER 4

KETAMINE HYDROCHLORIDE

INTRODUCTION

Ketamine has been a widely used anesthetic in pediatrics,[103] obstetrics,[5] and veterinary medicine.[82] The versatility of ketamine,[21,26] in addition to positive hemodynamic profile,[23,29] analgesic effects,[73] and low cost[19, 27,29] contributes to its continued use in humans[19] despite some associated undesirable emergence reactions.[28] The psychomimetic effects[28,31,32] of ketamine in combination with the abundant availability of ketamine contributes to its prevalent abuse.[7,11,29,101,116] The widespread use of ketamine and its potential for abuse make necessitates investigation of ketamine effects on embryonic and fetal development.

Ketamine easily crosses the placenta and is rapidly distributed in the fetal tissues,[31,64] likely due to its relatively low molecular weight.[31] Ketamine has also been found to produce uterine artery vasoconstriction, a fall in uterine blood flow, and eventual fetal deterioration,[12] and umbilical cord ketamine concentrations have been found to be higher than maternal blood concentrations.[31] Ketamine administration produces stimulating effects on the fetus, elevating fetal arterial pressure and heart rate;[64] however, no evidence of fetal hypoxia, metabolic acidosis, or arrhythmia has been found following ketamine application.[12]

Documentation of ketamine developmental defects has been limited to post-implantation development.[7,11] Toxicity of ketamine in the preimplantation embryo is previously unreported. Observations of ketamine developmental effects in CF-1 mice have shown daily 50mg/kg doses from gestation day 6-15 to produce insignificant effects on skeletal and visceral anatomy, as well as no increased resorptions, dead fetuses, or decreased fetal weight.[7] Further histological analysis of the heart, liver, and kidney effects, however, has revealed focal nuclear hypochromatosis, interfibrillary edema, parenchymal cell degeneration, and proximal convoluted tubule degeneration,[7,11] and degenerative effects were found to be dose and treatment duration dependent.[11]

The focus of this study was to evaluate preimplantation embryotoxicity of ketamine HCl. Toxicity of long-term exposure to ketamine HCl in the 2-cell preimplantation embryo is previously unreported. The window during preimplantation development, and the dosage and severity of embryotoxic effects which may be exerted by ketamine HCl has not been previously evaluated. Early embryonic stages provide indications of potential induction of fetal and postnatal defects,[157] and extended exposure allows determination of effects of later preimplantation development since some components have the ability to affect the development of the blastocyst and inner cell mass directly.[151] Extended exposure is valuable for the evaluation of potential embryotoxicity of pharmaceutical agents subject prolonged use or habitual

abuse, and analysis of extended effects is necessary to assess the effects of compounds which may compromise embryo development, causing failure of the bioassay without being outright lethal.[151]

This study was performed with the objective of evaluating the potential of ketamine HCl to impair cleavage through the normal progression of in vitro embryonic development from the 2-cell to the blastocyst stage, and the developmental timing and extent to which damage may be exerted. Potential adverse effects of ketamine HCl were assessed by individual culture of preimplantation 2-cell murine embryos with incremental concentrations of ketamine HCl, starting with a range encompassing and exceeding normal clinical concentrations, and surpassing the normal clinical duration of exposure. When extreme embryotoxicity was realized at concentrations exceeding the normal clinical ED during preliminary experimentation, the dosage of ketamine HCl was modified to a range of moderate effects near the relevant anesthetic dose in mice (50-100mg/kg, or 50-100μg/mL)[105] for follow up experiments. Embryotoxicity was assessed by analysis of early cleavage and subsequent differentiation of 2-cell preimplantation embryos subjected in vitro to prolonged 72h exposure of ketamine HCl. Detection of abnormal embryo morphology including fragmentation, granulation, and degeneration of blastomeres provided indications of deleterious clastogenic damage capable of significantly inhibiting in vitro development.

METHODS

Animals

Embryos used in the study were produced on IACUC protocol #12-017. Embryos were obtained from female mice 6-18 weeks old (B_6CBAF_1F, purchased from Jackson Laboratories, Bar Harbor, Maine) crossed with outbred CD-1 males 2-10 months old (Harlan Lab, Indianapolis, IN). This F2 hybrid mouse strain has proved to produce consistently high numbers of embryos with 100% development to blastula stage in 72h if all culturing variables are at an optimum. Mice were 4-6 weeks old when received and acclimated to the animal facility for at least 48h before use and were used within 12 weeks of purchase. Mice were maintained on a lighting regime of 14h light, 10h dark at 21°-25°C and given water and laboratory rodent chow (Harlan) *ad libitum*. Euthanasia was performed by cervical dislocation, approved by the Panel on Euthanasia of the American Veterinary Medical Association.

Superovulation And Mating

Female mice were selected at random for exogenously driven superovulation. Superovulation was achieved by intraperitoneal (IP) injection of gonadotropic hormones. IP injection was performed through the lower right quadrant of the abdomen using a 1mL syringe with a ½ inch 30 gauge needle. Beginning four days prior to use, the injection cycle consisted of two injections separated by 48h. The first injection at 0h was 0.1mL of a 50 international units (IU)/mL (5 IU) of pregnant mare serum gonadotropin (PMSG) (SIGMA, G4877, 106K1171; St. Louis) in phosphate buffered saline (PBS). At 48h after PMSG injection,

0.1mL of a 50 IU/mL (5 IU) solution of human chorionic gonadotropin (hCG) (SIGMA, CG5, 075K1442; St. Louis) in PBS was administered. At the time of hCG injection, each female was caged with one proven fertile male CD-1 mouse. Female mice go into estrous and mate within 6h of hCG injection, with ovulation occurring between 10 and 12h after hCG injection. Between 8 AM and 10 AM (16h after hCG injection) the following morning, female mice were inspected for vaginal copulatory plugs and the result of the performance of each male was recorded. At this time mated and unmated females were segregated and marked appropriately with cage cards; only mated animals were used. Unmated animals were submitted to the same injection cycle after a two-week recovery period during which the pituitary gland is less sensitive to gonadotropins. Females cycled three times without successful mating were removed and euthanized by cervical dislocation.

Collection Of Embryos

Females with vaginal copulatory plugs were euthanized using cervical dislocation starting approximately 42h after the hCG injection (pregnancy day 0) and immediately prior to embryo collection. The abdomen of the mouse was entered by producing a small hole above the umbilicus then retracting the skin simultaneously down over the hind legs and over the forelegs in a process known as de-gloving. This technique leaves a sterile field for entry without any mouse hair and obviates use of antiseptic agents that may compromise embryo viability. Bilateral celiotomy was performed to expose the oviducts of pregnant animals. Salpingectomy was performed by gently stretching and tearing the mesosalpinx and mesometrium away from each oviduct and uterine horn, and then cutting between the oviduct and ovary at the cephalad end and the oviduct and the intramural junction at the caudal end with microsurgical scissors. The two oviducts were then placed in each sterile 35 x 10mm polystyrene culture dish (Nalge Nunc International) containing 2mL of modified Krebs-Ringer-bicarbonate (mKRB) medium (See Appendix 1). A Zeiss dissecting stereomicroscope (Eastern Microscope Co, Raleigh, N.C.) at 20x magnification, watchmaker forceps (Dumont and Fils, Switzerland) was used to slide the fimbriated end of the oviduct onto a 30-gauge (FineJect, Henke Sass Wolf) sterile needle attached to a 1 mL sterile syringe (Norm-Ject). Embryos were irrigated from the oviducts with medium lightly expressed from the syringe. Each animal can be expected to produce an average of 15 embryos per successful mating (successful mating is approximately 1 per 2 stimulated females = (½)).

Embryos

Individual morphologically normal (judged by oval, bilateral symmetry of the two blastomeres with narrow perivitelline space and presence of two small polar bodies) 2-cell embryos was evaluated at 32x magnification and collected from the petri dish with a 10µL micropipette (Eppendorf) fitted with a sterile tip (Avant) and aseptically dispensed into 96 well U-bottom culture plates (Falcon, 35-3077) with one embryo per well. Embryos were cultured in a NuAire Autoflow IR Water Jacketed CO_2 Incubator at 37°C, 5% CO_2 for 72h. Differentiation was evaluated every 24h at 40x and 100x using a Nikon Diaphot inverted

microscope. Each test medium was evaluated in triplicate cultures.

Solutions And Media

Media used was mKRB solution supplemented with 4mg bovine serum albumin (BSA) per mL (See Appendix 1) and conditioned at 37°C 18-24h. Ketamine HCl (100 mg/mL) (Hospira, Inc. RL-0065) was dissolved in mKRB media at various concentrations and equilibrated for at least 12h before use. Control solutions included mKRB media for the ketamine HCl experiments, since during preliminary experiments the positive control containing the vehicle (0.1mg/mL benzethonium chloride pH 3.5-5.5) did not produce a significantly different result from media solution. Exploratory study was performed with an array of concentrations below and exceeding the relevant anesthetic dose (25-100mg/kg, or 25-100μg/mL for a 25g mouse) at 1.0, 10, 50, 100, 500, 1000, and 5000μg/mL ketamine HCl. A second set of experiments testing 20, 40, 60, 80, 100, 250μg/mL ketamine HCl was then performed to test the range below 100% toxicity where inhibition was present.

Statistics

A 72h embryo culture endpoint with minimum development of 65% blastula and combined 80% morula + blastula embryo morphology is necessary to report a passing score on the mouse embryo assay.[251] Cell number and overall developmental stage of embryos in specific treatments were analyzed using one-factor fixed-effects randomized block analysis of variance (ANOVA) performed using IBM SPSS 20 to test for overall significance among treatments. If significance was found, this was followed by Tukey's HSD (honestly significant difference) test (for unequal sample sizes in treatment groups) for differences between means of specific treatments. The Shapiro-Wilk test of normality and Levene's test of equality of variance were also used to evaluate whether samples were normally distributed and if there was statistically significant variation between treatments. The significant groups were then tested with χ^2 for homogeneity and difference of proportions. Culture of at least 15 embryos is performed with each item tested to meet the CLIA '88 federal law to establish toxicity for the tested item. This embryo number (15) was established by the R.J. Swanson Mouse Embryo Laboratory as the standard for their testing protocol.

RESULTS

The results of ketamine HCl experiments are presented in Table 5 and Table 6. Ketamine HCl did not produce any significant inhibitory effects on embryonic development up to and including a concentration of 50μg/mL during initial studies. At all concentrations greater than 50μg/mL ketamine HCl, significant inhibition of differentiation was observed, with fragmentation and degeneration in 100% of embryos cultured at concentrations exceeding 100μg/mL. All concentrations cultured at greater than 50μg/mL ketamine HCl resulted in a failing mouse embryo assay morula + blastocyst embryo score. The 0ng/mL ketamine HCl, 0.1mg/mL benzethonium chloride pH 3.5-5.5 control solution produced effects not significantly different from control mKRB media, and for follow up experimentation mKRB media was

used as the control solution. During preliminary experimentation a wide array of concentrations including a serial dilution of ketamine HCl was evaluated. This was performed with the objective of determining a more precise dosage range in which moderate toxic effects were exerted to allow more detailed analysis for follow-up experimentation. At 5000μg/mL ketamine HCl, 100% of embryos exhibited a 2-cell block and degenerated at the 2-cell stage without completing any cleavage divisions. At concentrations of 1000μg/mL 500μg/mL, and 250μg/mL ketamine HCl, 100% of embryos tested degenerated before reaching the blastocyst stage, however within these groups a significant percentage (13.3%) of embryos were able to complete at least one cleavage division (Table 5; Fig. 13) ($\chi 2$, $p<0.001$).

In the second set of experiments, the concentrations of ketamine HCl dosages were modified to the area within which inhibition was present but not 100% toxic to development (1.0-100μg/mL ketamine HCl). Ketamine HCl dosage increments of 20μg/mL were investigated, ranging from 0ng/mL-100μg/mL, and significant inhibitory effects were recorded at concentrations ≥60μg/mL (Table 5; Table 6). Significant degeneration and visible nuclear fragmentation was recorded for 31.5% of embryos treated at 100μg/mL ketamine HCl ($\chi 2$, $p<0.01$). Although the degeneration observed at 100μg/mL ketamine HCl was significant, 67% of embryos within the degenerating/fragmenting group (21% total) were able to complete several cleavage divisions to arrest between the 8-cell stage and just prior to morula by the 72h endpoint (Fig. 13; Fig. 14). This suggests that ketamine HCl inhibitory effects on differentiation may not be limited to the first cleavages, but also affects later stages of differentiation. Later inhibition was supported by the significant percentage of embryos reaching the compact morula stage (26.3%) and blastocyst stage (42.1%) at 100μg/mL ketamine HCl. At 80μg/mL ketamine HCl, significant degeneration and nuclear fragmentation was detected in 33.3% of embryos ($\chi 2$, $p<0.001$). Similar to the results of the 100μg/mL group, 60% of degenerating embryos (20% total) completed at least one cleavage division (Fig. 13; Fig. 14). By the 72h endpoint, a significant percentage of embryos at 80μg/mL reached the morula (40%) or blastocyst stage (26.7%), however the combined total resulted in a failing embryo score for this concentration.

The 60μg/mL ketamine HCl concentration did not exert effects causing degeneration or fragmentation, all embryos at this concentration reached the morula or blastocyst stage. A significant effect was found between embryos reaching the morula stage (40%) and those reaching the blastocyst stage (60%) ($\chi 2$, $p<0.01$), though, suggesting that 60μg/mL ketamine HCl may have an inhibitory effect to delaying later differentiation of the preimplantation embryo. This concentration was also the lowest dosage to result in a failing morula + blastocyst embryo score. At 40μg/mL ketamine HCl a significant effect ($\chi 2$, $p<0.01$) was found between embryos reaching the blastocyst stage (66.6%) and those delayed at the morula stage (26.7%). A significant inhibitory effect was also present at the lowest concentration tested, 1.0μg/mL ketamine HCl, where only 66.7% of embryos at this stage were able to reach the blastocyst stage, 13.3% reached the compact morula stage, and 20% degenerated at the 4-cell to 12-cell stage (6.7% at 4-cell). Inhibition of development to blastocyst was significant at 1.0μg/mL ($\chi 2$, $p<0.05$), although this

concentration yielded a passing embryo score. At the next highest concentration, 10µg/mL, 93.3% of embryos reached the blastocyst stage with 6.7% exhibiting a 2-cell block.

Results of the one-factor fixed-effects randomized block ANOVA for overall significance among treatments found a significant effect between developmental stage groups (degenerating/fragmenting, compact morula, and blastocyst) ((F=3.988; d*f*=2,36; P=0.0273), however a significant difference between concentrations was not found. This analysis was followed by Post Hoc Tukey's HSD test to evaluate differences between specific treatments. The Tukey's HSD test did not reveal a significant effect between specific blocks, except between the morula and blastocyst groups (P=0.025). The Shapiro-Wilk test of normality indicated that the samples were not normally distributed (P=0.003, 0.005, 0.010). Levene's test of equality of variance was statistically significant (P=0.001), indicating a significant effect on differentiation by ketamine HCl.

Table 5. Ketamine hydrochloride 72h effects on embryonic development: differentiation percentage of embryos

Ketamine HCl µg/mL	2-Cell	4- 12-Cell	Total Degenerating	Morula	Blastocyst	Total Cultured
0 (Control)	0	0	0	12.50%	87.5%	32
1.0	0	20%	20%	13.30%	66.70%	15
10	6.70%	0	6.70%	0	93.30%	15
20	12.50%	0	12.50%	6.25%	81.25%	16
40	6.70%	0	6.70%	26.70%	66.60%	15
50	0	0	0	6.70%	93.30%	15
60	0	0	0	40%	60%	15
80	13.30%	20%	33.30%	40%	26.70%	15
100	10.50%	21%	31.50%	26.30%	42.10%	19
250	86.70%	13.30%	100%	0	0	15
500	86.70%	13.30%	100%	0	0	15
1000	93.30%	6.70%	100%	0	0	15
5000	100%	0	100%	0	0	15

Ketamine HCl buffered with mKRB media + BSA was tested at the above concentrations to ascertain potential for embryotoxicity. Degenerating refers to abnormal development or deterioration of the embryo before the compact morula stage. Control solution contained mKRB+BSA embryo culture media sans ketamine HCl.

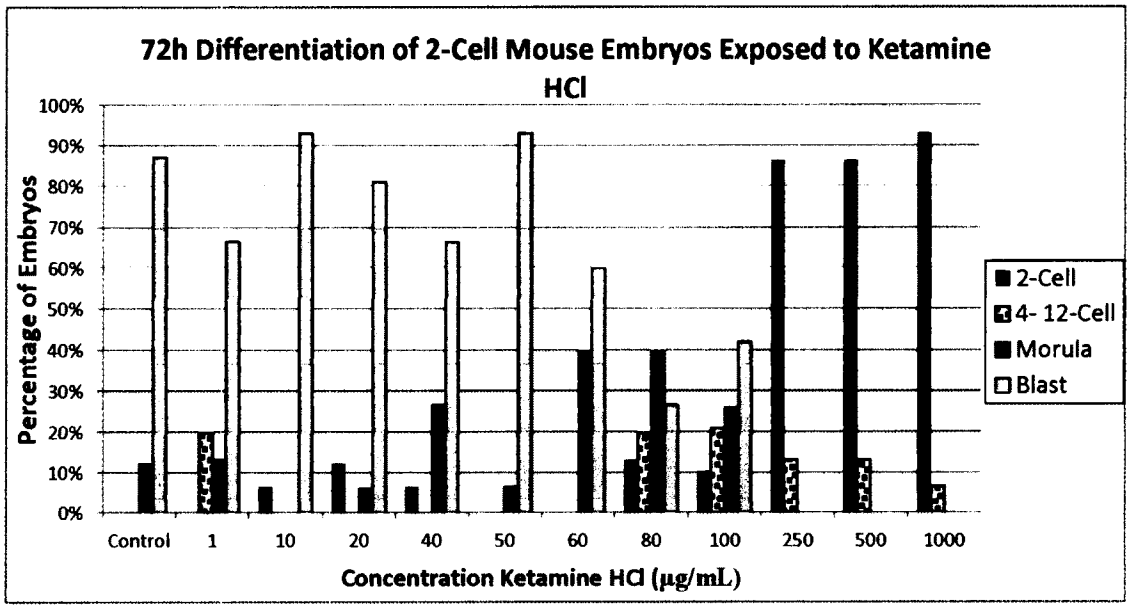

Fig. 13. 72h differentiation of 2-cell mouse embryos exposed to ketamine hydrochloride. Effects of ketamine HCl indicated by percentage of embryos reaching specific stage of differentiation at 72h. Ketamine HCl buffered with mKRB media+BSA was tested at the above concentrations. Control solution contained mKRB+BSA embryo culture media sans ketamine HCl. Blast refers to blastocyst (32+ cells).

Table 6. Ketamine hydrochloride 72h effects on embryonic development: percentage of embryos reaching specific stage of differentiation

Ketamine HCl (µg/mL)	2-Cell	4-Cell	8-Cell-12-Cell	Total Degenerating	Morula	Early Blast	Hatching Blast
0 (Control)	0%	0%	0%	0%	12.5%	65.6%	21.9%
1	0%	6.7%	13.3%	20%	13.3%	20%	46.7%
10	6.7%	0%	0%	6.7%	0%	20%	73.3%
20	12.5%	0%	0%	12.5%	6.25%	50%	31.25%
40	6.7%	0%	0%	6.7%	26.7%	53.3%	13.3%
50	0%	0%	0%	0%	6.7%	13.3%	80%
60	0%	0%	0%	0%	40%	46.7%	13.3%
80	13.3%	6.7%	13.3%	33.3%	40%	26.7%	0%
100	10.5%	0%	21%	31.6%	26.3%	31.6%	10.5%
250	86.7%	6.7%	6.7%	100%	0%	0%	0%
500	86.7%	0%	13.3%	100%	0%	0%	0%
1000	86.7%	6.7%	6.7%	100%	0%	0%	0%
5000	100%	0%	0%	100%	0%	0%	0%

Ketamine HCl buffered with mKRB media + BSA tested at the above concentrations to ascertain potential for embryotoxicity. Degenerating refers to abnormal development or deterioration of the embryo before the compact morula stage, Blast refers to Blastocyst. Control solution contained mKRB+BSA embryo culture media sans ketamine HCl.

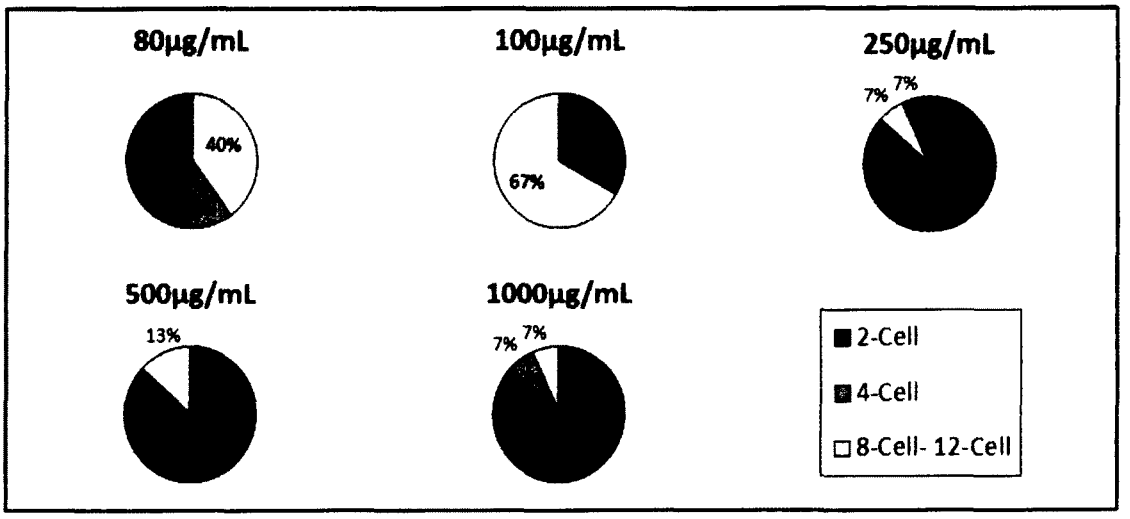

Fig. 14. Differentiation progress of degenerating embryos following 72h exposure to ketamine hydrochloride. Percentages of embryos arrested at the 2-cell stage in comparison to embryos able to undergo at least one cellular division before degenerating and/or fragmenting (4-Cell and 8-Cell-12-Cell).

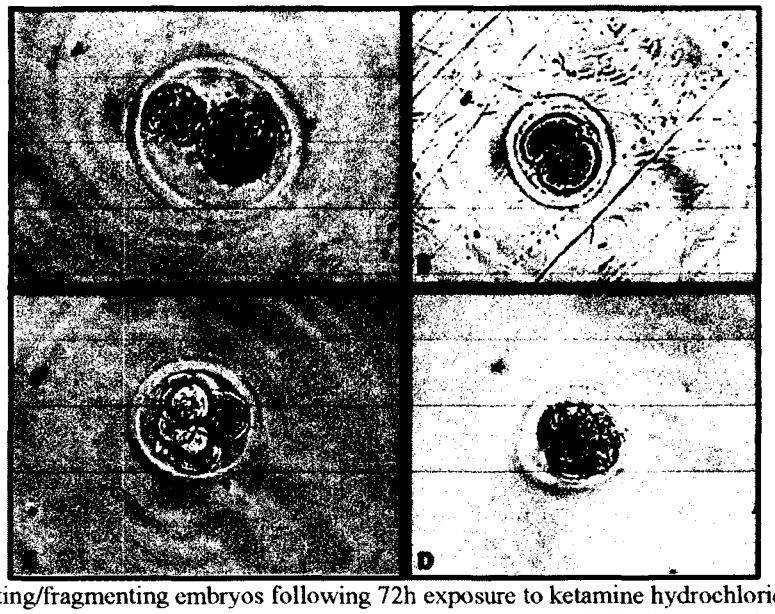

Fig. 15. Degenerating/fragmenting embryos following 72h exposure to ketamine hydrochloride. Composite image of degenerating/fragmenting mouse embryo morphology following 72h of culture at 1000µg/mL ketamine HCl (A) and (B), 500µg/mL ketamine HCl (C), and 250µg/mL ketamine HCl (D). (A) Irregular shape of blastomeres and cellular fragments are indications of extensive degeneration and apoptosis. (B) Very similar appearance to that of normal 2-cell embryo, however blastomeres have a more granulated appearance than a normal 2-cell embryo. At 72h this morphology is demonstrative of the "2-cell block." (C) 6-8-cell embryo, exhibiting irregular cleavage, and minor fragmentation. (D) 8-cell embryo with more compact organized structure than (C), however blastomeres are slightly granulated and fragments are visible within the zona pellucida at right. Photomicrographs taken using DIC Optics at 200x.

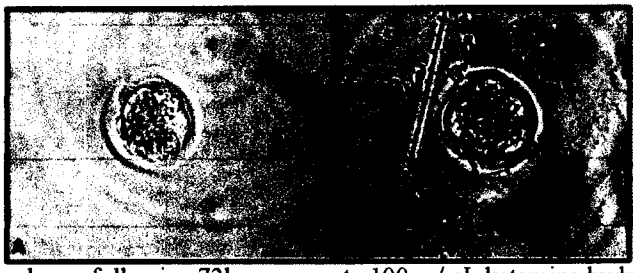

Fig. 16. Morula stage embryos following 72h exposure to 100µg/mL ketamine hydrochloride. Photomicrograph taken using DIC Optics at 200x.

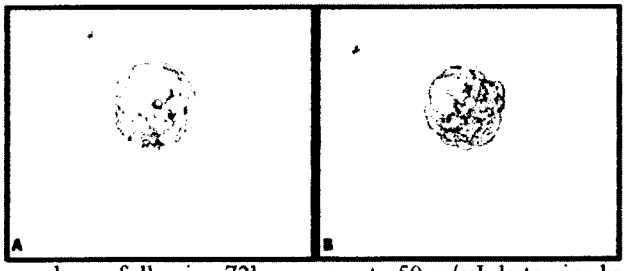

Fig. 17. Blastocyst stage embryos following 72h exposure to 50µg/mL ketamine hydrochloride. Expanded blastocyst (A) and early blastocyst (B) both categorized as early blastocyst for statistical analysis.

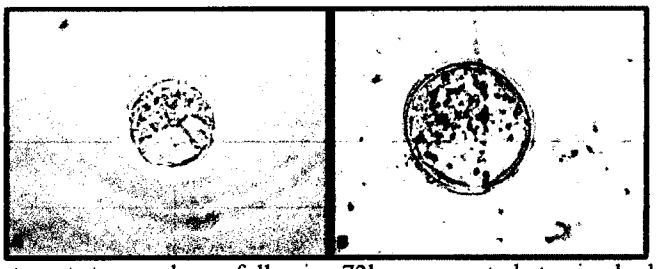

Fig. 18. Expanded blastocyst stage embryos following 72h exposure to ketamine hydrochloride. Effects of 20µg/mL (A) and 10µg/mL (B) ketamine HCl indicated above.

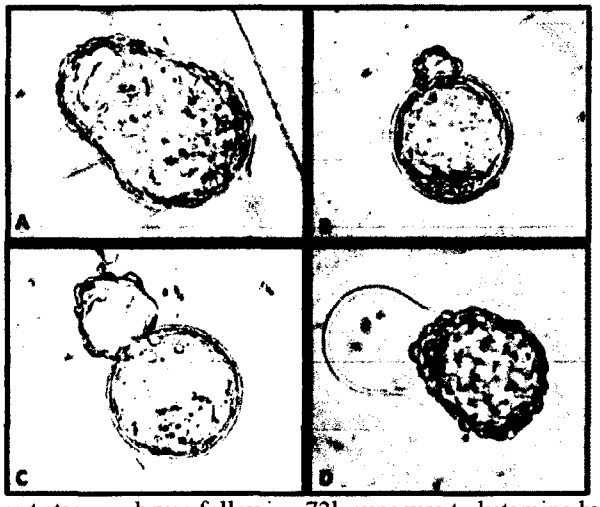

Fig. 19. Hatching blastocyst stage embryos following 72h exposure to ketamine hydrochloride. Blastocyst stage embryos "hatching" from the zona pellucida following 72h culture at 1.0μg/mL ketamine HCl (A) and (B), and control mKRB media + BSA (C) and (D).

DISCUSSION

The results of this study suggest that preimplantation murine embryos exposed to ketamine HCl in extended in vitro culture exhibit dose-dependent inhibitory effects. Statistical analysis was initially performed for embryo morphology groups used for toxicity evaluation in the 72h mouse embryo bioassay (degeneration, morula, and blastocyst). Follow-up χ^2 tests for homogeneity and difference of proportions to analyze specific differentiation stages following completion of experimentation found compelling effects. Ketamine HCl completely inhibited differentiation to the blastocyst stage at high concentrations (250μg/mL, 500μg/mL, 1000μg/mL, and 5000μg/mL). At these concentrations, significant embryotoxicity was observed within 48h of culture and affected the first cleavages, and 100% of embryos arrested and degenerated before reaching the morula stage. Detailed analysis of the degeneration within these groups, however, showed 5000μg/mL ketamine HCl to be the only concentration to induce a 2-cell block in 100% of embryos. A significant percentage of embryos at 250μg/mL, 500μg/mL, 1000μg/mL were able to complete at least one cleavage division before arresting ($\chi 2$, $p<0.001$). At lower concentrations (≤50μg/mL), ketamine HCl produced no significant inhibitory effects on embryonic development (Table 6), with the exception of 1.0μg/mL. Since effects of ketamine HCl on in vitro preimplantation embryo differentiation are previously unreported, testing of a wide array of concentrations was necessary to determine a more precise range for follow-up experimentation. The concentrations of ketamine HCl dosages were modified to include the range found to be inhibitory but not prohibitive to differentiation to the blastocyst stage (0-100μg/mL) in exploratory experiments. The coincidence that inhibitory effects were observed in embryos cultured near the range of the anesthetic ED for mice (25-100mg/kg, or 25-100μg/mL) is significant and suggests a concomitant effect.

Significant degeneration and visible nuclear fragmentation was present in 31.5% of embryos treated with 100μg/mL ketamine HCl. While significant degeneration was observed, only 10.5% of embryos at this concentration were arrested at the 72h endpoint, with 21% able to undergo at least one cellular division (Fig. 13; Fig.14). This result suggests that the inhibition of differentiation was not limited to a cleavage block at the 2-cell stage, since a significant percentage was able to complete several cleavage divisions before arresting ($\chi 2$, $p<0.001$). Signs of embryonic delay were also displayed by embryos treated at this dosage, with a significant percentage (26.7%) reaching the compact morula stage, and 42.1% reaching the blastocyst stage ($\chi 2$, $p=0.0062$). At 80μg/mL ketamine HCl, significant degeneration and visible nuclear fragmentation was detected in 33% of embryos ($\chi 2$, $p<0.001$), however a significant difference between degenerating, morula, and blastocyst groups was not detected. Further analysis found similar results to those of the 100μg/mL ketamine HCl group, 20% of embryos tested (60% of those degenerating, Fig. 14) were able to complete at least one cleavage division ($\chi 2$, $p=0.0217$). Analysis of effects on later cleavage found no significant effect between embryos reaching morula, early blastocyst, or hatching blastocyst stages. Both 100μg/mL and 80μg/mL ketamine HCl concentrations resulted in similar failing morula + blastula embryo scores (68.5% at 100μg/mL; 66.7% at 80μg/mL ketamine HCl).

Embryos cultured at 60μg/mL ketamine HCl exhibited no significant degeneration, visible nuclear fragmentation, or indication of early delay or arrest before the morula stage. Although 100% of embryos at 60μg/mL ketamine HCl were able to reach at least the morula stage, similar to higher concentrations, a failing embryo score resulted since less than 65% reached the blastocyst stage.[251] In addition, a significant effect was found between embryos reaching the morula stage (40%) and those reaching the blastocyst stage (60%) ($\chi 2$, $p=0.0455$), as well as between those reaching morula, early blastocyst, or hatching blastocyst ($\chi 2$, $p<0.001$). This result suggests that 60μg/mL ketamine HCl exerts inhibitory effects to slow later stages of preimplantation embryonic development. At all concentrations below 60μg/mL ketamine HCl, a passing embryo score was calculated. Significant differences between degenerating, morula, and blastocyst groups were calculated within this range, as well as significant differences between later differentiation stages ($\chi 2$, $p<0.001$). Although the lowest concentration tested resulted in a passing embryo score, 1.0μg/mL ketamine HCl induced a significant degeneration in 20% of embryos ($\chi 2$, $p<0.001$). At this concentration, no embryos were blocked at the 2-cell stage, however 20% were arrested between the 4-cell and 12-cell stages, 13.3% reached the compact morula stage, and 66.7% reached the blastocyst stage. While this effect was significant, the early inhibition may not necessarily be attributed to exposure to ketamine HCl, since 93.3% of embryos reached the blastocyst stage at the next highest concentration tested, 10μg/mL. Problems with culture medium preparations which fail to support adequate embryo development are often traced to technician error,[141] however only 30-50% of human embryos reach the blastocyst stage in vitro, with causes commonly attributed to chromosome abnormality and genetic defects.[178] In addition, it is not uncommon for a few 2-cell embryos to fragment or degenerate during culture with Kreb's media specifically prepared for toxicity testing and culture of mouse embryos.[144] These

effects may potentially account for the early fragmentation and degeneration found at 1.0μg/mL, 10μg/mL, 20μg/mL, and 40μg/mL ketamine HCl, and conceivably the inhibition of some embryos at higher concentrations (>60μg/mL).

The embryotoxicity of ketamine HCl exposure in the current study may be attributed to a variety of biochemical mechanisms or a combination of toxic effects. Exogenous compounds can affect development of the preimplantation embryo by increasing oxidative stress, and due to relatively weak antioxidant defense during early developmental stages,[227] this stress often results in severe embryonic damage or arrest.[190,224,227] Oxidative stress has previously been found to produce a developmental block in 2-cell mouse embryos,[224] and is a likely factor contributing to impeded differentiation in embryos cultured at concentrations above 60μg/mL ketamine HCl in this study. Oxidative stress is also capable of affecting development via numerous secondary effects such as directly causing DNA or RNA damage,[190,227] as well as affecting nutrient uptake and metabolism,[198] mitosis,[236] and apoptosis.[178] Oxidative stress may be intensified by autocatalytic effects; free radicals can damage mitochondrial membranes and generate new sources of ROS.[198] Environmental agents that elevate levels of free radicals may also affect the developing embryo by increasing lipid peroxidation and protein oxidation.[227] Ketamine has previously been found to increase ROS production, induce expression of oxidative stress-related genes, and dose- and time-dependently cause cell death in other tissues;[103] similar effects may be exerted by ketamine HCl in the preimplantation embryo.

Induced expression of HSPs due to suboptimal culture conditions and constitutive HSP expression both represent an essential mechanism for successful embryo growth in an adverse environment, however inducible expression is not active until the morula or early blastocyst stage.[178] A cellular response to oxidative stress or chemical insult is increased expression of HSPs.[225] HSPs are regulated by heat shock factors,[170,246] but loss of heat shock factor function has previously been found to result in developmental arrest as early as the 2-cell stage.[197,225,246] Inadequate HSP expression at the time of initial ketamine HCl contact may have contributed to inability of 2-cell embryos to recover from the chemical insult of exposure at >60μg/mL ketamine HCl and arrest during early cleavage. Early HSP expression may have also assisted in the survival allowing additional cleavage divisions of embryos not blocked at the 2-cell stage if this concentration did not overwhelm the minor protective effects of early HSP expression.

Inducible HSP expression may have also contributed to the delay of later development in embryos cultured at lower concentrations of ketamine HCl (≤60μg/mL), where inhibited formation and maturation of blastocysts occurred without significant interference to cleavage prior to compaction. Induced expression of HSPs up regulated during the morula to blastocyst stage may have significantly assisted resistance to ketamine HCl to allow later cleavage and blastocyst formation, possibly at concentrations up to 100μg/mL. The significant percentage of embryos delayed at the morula stage or early blastocyst stage at concentrations between 1.0 and 100μg/mL ketamine HCl (except 80μg/mL) also supports this

assumption, however below 40μg/mL effects of inhibition of blastocyst do not significantly differ from those recorded in control solutions.

The ability of a significant percentage embryos to complete at least one cellular division before degenerating at concentrations up to 1000μg/mL ketamine HCl demonstrated that even at high concentrations, some embryos were able to endure toxic effects long enough to stagger through at least one additional cleavage. This capacity for survival was especially significant at concentrations ≤100μg/mL ketamine HCl, and may be attributed to moderate toxicity at lower concentrations only affecting select cellular processes, perhaps in combination with the capacity of embryonic endogenous mechanisms such as HSPs (even basal expression) to preserve some normal functions. Embryonic repair may also have assisted survival; undifferentiated cells of the early embryo make repair possible through multiplication of cells,[4] even if development may still be compromised by non-lethal insults.[151,224]

Blastomeres of embryos cultured at concentrations above 60μg/mL ketamine HCl displayed substantial fragmentation indicative of clastogenic damage and apoptosis likely contributing to significant inhibition of early cleavage. This result is consistent with results previously found with another anesthetic NMDA antagonist nitrous oxide,[66] where 2-cell, 4-cell, and morula stage embryos were exposed to doses of nitrous oxide determine effects on subsequent development following short-term exposure.[2] All groups were exposed for 30 minutes, but significant effects were only observed in 2-cell embryos treated within 4h of expected cleavage. Exposure of the 2-cell embryos to nitrous oxide resulted in significant inhibition of blastocyst development compared to control groups. In addition, the majority of affected embryos were halted at the 2-cell stage with no further cellular division,[2] comparable to the significant 2-cell block recorded for embryos cultured at concentrations greater than 100μg/mL ketamine HCl in this study. Cellular fragmentation characteristic of apoptosis was significant during early cleavage of embryos exposed to concentrations greater than 60μg/mL ketamine HCl, and increased apoptosis or an imbalance of apoptotic proteins may have also contributed to delayed differentiation observed during later cleavage of embryos cultured between 1.0μg/mL and 100μg/mL ketamine HCl (with the exception of 80μg/mL). Multiple stimuli such as oxidant insults, pro-apoptotic BAX protein activation, intracellular Ca^{2+} overload, and direct activation of other caspases are capable of activating caspase enzymes to initiate apoptosis,[173] and in vitro induction of apoptosis may be attributed to suboptimal culture conditions, excessive ROS exposure, or chromosomal abnormalities.[178] High levels of apoptosis have previously been detected in fragmenting preimplantation embryos,[238] however determination of the mechanism responsible for induction of apoptosis in this study requires further investigation.

Alteration of the redox-balance has the potential to induce oxidative stress-mediated damage to mitochondrial proteins and result in mitochondrial dysfunction.[198] Ketamine has previously been found to have significant deleterious mitochondrial effects in neurons, including induced mitochondrial ultrastructural abnormalities, decreased inner mitochondrial membrane potential ($\Delta\Psi m$), and increased mitochondrial cytochrome c release, resulting in toxicity by the mitochondrial apoptotic pathway.[103]

Ketamine HCl may also have profound effects on mitochondrial function in the preimplantation embryo, which would significantly contribute to the inhibition of differentiation observed in this study.

Mitochondrial function is critical for the progression of early development,[190] and mitochondrial damage or alteration of membrane and intracellular proteins can perturb metabolism or many other cellular processes such as oxidative phosphorylation and provision of intermediates for glycolysis, affect ion homeostasis, signal transduction, and apoptosis.[196] In addition to initiation of apoptosis, disruption of mitochondrial function would affect metabolism during pre-compaction stages, when energy production is nearly entirely dependent on the mitochondrial TCA cycle.[179,188,189] Damage from oxidative stress has previously been found to cause cells to shift from oxidative use of pyruvate in the full TCA cycle to use of the segment of the TCA cycle involving succinate and succinate dehydrogenase.[198] Alteration of the TCA cycle can significantly interrupt the main mechanism of ATP production during early embryonic differentiation, potentially contributing to the significant inhibition and degeneration during the first cleavage divisions in embryos cultured at concentrations greater than 60μg/mL ketamine HCl. The significantly delayed differentiation and arrest of cleavage prior to reaching the blastocyst observed in embryos cultured at concentrations between 60-100μg/mL ketamine HCl may also be attributed to interrupted metabolism by ketamine-induced mitochondrial damage. ATP is necessary for embryonic compaction between the 8-cell and morula stage,[173] as well as for active transport to internalize fluid for formation of the blastocoel.[165,169,171] Expansion of the blastocoel is essential for ICM differentiation as well as for embryonic hatching from the zona pellucida.[152,165,174] Later delayed differentiation observed in embryos at lower ketamine concentrations may also be attributed to metabolic effects resulting from mitochondrial dysfunction, depriving embryos of necessary ATP for mitotic divisions and blastocyst differentiation; however the trophectoderm Na^+/K^+ pumps necessary for blastocoel expansion may have also been affected by ATP deficiency.[173] Ketamine-induced oxidative damage to mitochondrial membranes may have also contributed to later inhibition by depriving embryos of glycolysis intermediates.

Embryonic dependence on the TCA cycle during pre-compaction stages shifts to a metabolism based on glycolysis between the morula and blastocyst stages.[179,188,189] Prior to compaction, glucose is not utilized as the main energy source,[166] but between the 8-cell to morula stage, glucose consumption increases dramatically[179,190] and glucose is utilized as the primary energy substrate by the blastocyst stage.[166] Glucose uptake has previously been found to be interrupted by ketamine,[48] and the inhibitory effect of ketamine HCl on glucose transport may be involved in the delay of later differentiation between the morula and hatching blastocyst stages observed in this study. Although the TCA cycle is dominant during early cleavages, the glucose transporter GLUT1 is has been detected at every stage of development and is believed to satisfy basal glucose requirements of the embryo.[193] In addition, interference with facilitators of glucose uptake such as GLUT1 have previously been shown to result in a high rate of apoptosis during the blastocyst stage.[244] The inhibition of GLUT1 and associated decreased glucose transport previously found to be induced by ketamine may have a similar apoptotic effect on the mouse

embryo. Once embryonic metabolism becomes dependent on glycolysis, ketamine HCl induced inhibition of glucose transport may significantly slow cellular processes and result in arrest before the blastocyst stage similar to that observed in embryos cultured at 40μg/mL, 60μg/mL, 80μg/mL, and 100μg/mL. Increased apoptosis resulting from interference with facilitators of glucose uptake by ketamine HCl may have also contributed the later inhibition at 40μg/mL, 60μg/mL, 80μg/mL, and 100μg/mL.

In the embryo, the cell cycle can be modified either by introduction of new components, substrates, pathways, or altered interactions between them.[232] At different phases of the cell cycle, the embryo may be more resistant or sensitive to environmental factors. Inhibitory effects found in the highest concentration groups suggest there significant modification to the cell cycle, possibly due to differences in sensitivity to ketamine HCl at various points within the cell cycle during initial exposure. During the G1 phase of the cell cycle, preimplantation embryos are particularly susceptible to environmental factors.[15] The timing of teratogen exposure during the cell cycle is consequently very important to embryonic survival, and embryotoxic effects during early cleavage have previously been attributed to cell cycle differences.[15] At the time of initial ketamine HCl contact, affected embryos may have been at the sensitive G1 phase or at a transition in stages of the cell cycle, which could differ between embryos depending on the timing of insemination.[15] This could potentially contribute to the significant variation in sensitivity to ketamine HCl found at 80μg/mL and 100μg/mL (Table 6; Fig. 13; Fig. 14).

Antimitotic effects resulting from ketamine HCl exposure to induce the 2-cell block in embryos at 80μg/mL and 100μg/mL ketamine HCl, and especially 2-cell block of embryos between 250-5000μg/mL ketamine HCl could be exerted by a number of mechanisms. Cell cycle control proteins Cdc25 phosphatases[235] may be inactivated by ROS, which has been found to lead to a block of the cell cycle progression.[236] Ketamine HCl may also have induced improper attachment of kinetochores to microtubules, which would trigger the spindle assembly checkpoint (SAC).[210] Activation of the SAC can prevent the onset of anaphase[210] to arrest the cell cycle. At higher concentrations, especially above 100μg/mL ketamine HCl, the SAC likely contributed to the arrest and subsequent degeneration of 2-cell embryos. Several local anesthetics (dibucaine, tetracaine, procaine, and lidocaine) have previously been found to significantly affect the organization of cytoskeletal components and plasma membrane attachment points[223] and similar effect may be produced by a general anesthetic such as ketamine HCl.

The preimplantation embryo is particularly sensitive to oxidative stress during ZGA, and any condition which enhances ROS accumulation during ZGA can have detrimental consequences.[190] Failure to undergo ZGA often results in arrested progression,[170,177] comparable to the 2-cell block of embryos exposed to 80μg/mL, 100μg/mL, and especially between 250-5000μg/mL ketamine HCl. Chromatin remodeling, a process requiring competent transcription factor function, is necessary for successful ZGA and embryonic differentiation,[170] and defective transcription factor function can result in developmental arrest.[197,225,246] Culture media has previously been found to directly affect gene expression (actin, G-3PDH, and Na^+/K^+ATPase housekeeping genes), transcription factors, and growth factors, as well as impacting the rate

of degradation of mRNAs which can affect methylation and expression.[228] Interference with chromatin remodeling or transcription factors may have been another contributor to the 2-cell block of embryos in higher concentrations. The zygote and the 2-cell embryo are also susceptible to certain mutagens with a delayed expression of damage,[157] which may have contributed to the embryonal delay observed in embryos at 60μg/mL, 80μg/mL, and 100μg/mL ketamine HCl. Future experimentation is necessary to determine whether the higher doses of ketamine HCl (>60μg/mL) induced a direct mutation of maternal-effect genes and affected or prevented ZGA, or oxidative stress exerted by any of these concentrations affected ZGA to delay differentiation or cause a developmental block at the 2-cell stage.

The results of this study suggest that exposure to ketamine HCl near and exceeding clinical concentrations affects preimplantation mouse embryo development by delaying or arresting early cleavage. Ketamine HCl at concentrations within the range of the clinical ED (≥60μg/mL ketamine HCl) significantly inhibited in vitro differentiation to the blastocyst stage. Concentrations below 60μg/mL ketamine HCl, however, did not significantly affect differentiation to the blastocyst stage. Further testing of concentrations below and within the range of relevant clinical dose of ketamine HCl (25-100μg/mL) should enable detailed analysis of potential molecular mechanisms contributing to the inhibition of preimplantation embryonic development by ketamine HCl.

Detailed examination of ketamine HCl effects on preimplantation embryo biochemistry is necessary to confirm that apoptosis is indeed activated by ketamine HCl exposure, that increased apoptosis contributed to inhibited differentiation, and to elucidate the mechanism(s) by which apoptosis is initiated. Additional mechanistic study is needed for characterization of the significant 2-cell block recorded at ketamine HCl doses exceeding 60μg/mL, and may find a correlation between either direct mutagenic effects or ketamine-exerted oxidative stress effects resulting in arrest of cleavage. Continued biochemical investigation of ketamine HCl effects in the preimplantation embryo should also provide insight into the mechanism by which inhibition is exerted. DNA and RNA damage, interference with transcription and transcription factors, suppression of HSP expression, antimitotic effects and spindle formation, mitochondrial interactions, initiation of the apoptotic cascade, interruption of metabolism and nutrient uptake, or a combination of any of these factors should allow a greater understanding of the inhibition caused by ketamine HCl. Investigation of later differentiation may also provide insight into how either increased apoptosis or ATP deprivation resulting in insufficient energy to sustain further mitosis or power Na^+/K^+ pumps necessary for blastocoel development may affect the inhibition of blastocyst hatching in progressively increasing ketamine HCl concentrations. Many of these factors may be investigated by experimentation in non-embryonic cells such as fibroblasts, mouse embryonic stem cells, mammalian micromass cell culture, or sperm cell viability, which would allow decreased expenses and animal use for abundant and timely data collection prior to testing effects in the preimplantation embryo. Future study of embryo transfer for implantation and fetal development may also provide evidence to support significant

effects of ketamine HCl on embryonic development does indeed exist and may potentially result in a decrease in live pup births (embryo or fetal resorption) or an increase in teratogenesis/birth defects.

CHAPTER 5

CONCLUSIONS

The potential teratogenic risk of anesthetic medications is difficult to assess in vivo.[5] Pregnant patients requiring surgery often have an infection or fever which may be detrimental to development in addition to hemodynamic changes and metabolic disturbances related to the surgery itself.[5] Data from in vitro assays of tissue, cellular and biochemical functions from animal models are useful for evaluation of human safety risks.[156] The limitations of extrapolating potential for human risks from in vitro and animal studies are largely attributed to lack of cross-species translation.[156] Differences in biological pathways and pharmacokinetics between species[156] combined with the complexity of mammalian reproduction, makes modeling and characterization of complete chemical effects on mammalian reproduction impossible.[144] In vitro culture may never be able to encompass all aspects of prenatal development; however the lack of validated alternative tests of embryonic development from gamete maturation to embryo implantation, makes animal testing and in vitro culture the only current option to assess the possible effects of exogenous agents to development.[149,162] Breaking down mammalian reproduction to biological components allows functions to be studied individually or in combination,[162] and isolation of components can assist in classification of chemicals as harmful to development.[149,162] Whether sufficient information can be derived from the results of these tests to label agents toxic to the developing embryo is debatable, and one must be cautious when attempting to translate the results of laboratory embryotoxicity studies to humans. Culture of the mammalian preimplantation embryo in vitro is associated with decreased viability,[254] however blastocyst morphology provides a good indicator of embryonic potential for implantation and subsequent development.[152] The results of the current study of the effects of prolonged exposure to propofol, NaPB, and ketamine HCl to early mouse embryonic development suggest potential risks during early pregnancy.

The results of this study also suggest that concentrations near the clinical ED for propofol and concentrations well below the ED of NaPB inhibit development of the preimplantation mouse embryo by delaying and arresting embryonic cleavage. The results of this study also suggest that exposure to ketamine HCl near and exceeding clinical concentrations delays or arrests early cleavage, and ketamine HCl at concentrations within the range of the clinical ED (≥60μg/mL ketamine HCl) significantly inhibited in vitro differentiation to the blastocyst stage. Ketamine HCl also exerted 100% inhibition of differentiation to blastocyst at concentrations above 100μg/mL. Lower test concentrations of all three compounds (≤ 3.0μg/mL NaPB, <60μg/mL ketamine, and <2.0μg/mL propofol), did not significantly affect differentiation to the blastocyst stage. Further testing of concentrations below and within the range of relevant clinical dose of propofol (human 1.5-2.5μg/mL, 1.5-2.5mg/kg), veterinary conentrations of ketamine HCl (mouse 25-100μg/mL, 25-100mg/kg), and below relevant concentrations of NaPB (0-300μg/mL, 0-300mg/kg depending on species) should enable detailed analysis and characterization of potential molecular

mechanisms contributing to the inhibition of preimplantation embryonic development by all three compounds.

Detailed examination of propofol, NaPB, and ketamine HCl effects on preimplantation embryo biochemistry is necessary to confirm that apoptosis is indeed activated by exposure to these anesthetics, that increased apoptosis contributed to inhibited differentiation, and to elucidate the mechanism(s) by which apoptosis is initiated. Additional mechanistic study is needed for characterization of the significant 2-cell block recorded at higher concentrations of these drugs, and may find a correlation between either direct mutagenic effects or drug-induced oxidative stress effects resulting in arrest of cleavage. Continued biochemical investigation of anesthetic effects in the preimplantation embryo should also provide insight into the mechanism by which inhibition is exerted. DNA and RNA damage, interference with transcription and transcription factors, suppression of HSP expression, antimitotic effects and spindle formation, mitochondrial interactions, initiation of the apoptotic cascade, interruption of metabolism and nutrient uptake, or a combination of any of these factors should allow a greater understanding of the inhibition caused by propofol, NaPB, and ketamine HCl. Investigation of later differentiation may also provide insight into how either increased apoptosis or ATP deprivation resulting in insufficient energy to sustain further mitosis or power Na^+/K^+ pumps necessary for blastocoel development may affect the inhibition of blastocyst hatching in progressively increasing anesthetic concentrations.

Probing for receptors or specific biochemical responses may be accomplished using radiolabelling with substances such as radioactive amino acids (e.g. [35S]methionine), however the radioactivity itself may damage chromatin, DNA, or other vital cellular components.[255]

Many of these factors may be investigated by experimentation with non-embryonic cells such as fibroblasts, mouse embryonic stem cells, mammalian micromass cells, or testing sperm cell viability. These tests would decrease expenses, time, and animal use to maximize data collection prior to testing effects in the preimplantation embryo. Results of these tests would serve as preliminary indicators of effects, not as a replacement for study of embryo culture; sufficient information may not be derived from alternative tests to allow classification of chemicals as toxic to embryonic development.[149,162]

Understanding the mechanisms underlying the inhibition induced by these drugs would greatly assist in the prevention of teratogenesis caused by exposure to these anesthetics.

Future study of embryo transfer for implantation and fetal development may also provide evidence to support significant effects of propofol, NaPB, or ketamine HCl on embryonic development does indeed exist and may potentially result in a decrease in live pup births (embryo or fetal resorption) or an increase in teratogenesis/birth defects. In addition to analysis of neonatal pups, ultrasound study of embryonic and fetal effects of anesthetic exposure at various developmental stages may provide valuable information of potential for teratogenesis. Selection of specific time points during development is necessary since real-time study of embryonic development is a difficult, tedious, and expensive endeavor, requiring use of more animals and equipment,[155] or collective culture of embryos which may also affect results. Additional study

of alternative agents such as thiopental, etomidate, IV anesthetic cocktails, and inhalation agents may also be performed once model experiments using propofol, NaPB, and ketamine HCl have concluded.

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APPENDIX 1

MODIFIED KREBS MEDIUM PREPARATION

Reagent	Amount
NaCl	0.5499g
KCl	0.0356g
$CaCl_2$ H_2O	0.0252g
KH_2PO_4	0.0161g
$MgSO_4$	0.0143g
NaHCO	0.2109g
Na Lactate	0.3920mL
Na Pyruvate	0.0055g
Glucose	0.1000g
BSA (Bovine Serum Albumin)	0.4000g
Pen/Strep	1.0 mL if 5000 IU

Osmolarity Range: 270-300 mOsm/kg H_2O
pH Range: 7.4-7.8

For preparation of 100mL mKREBS solution. After composition, media is checked for a pH value between 7.6 and 7.8 (before CO2 equilibration); filter-sterilized into 150 mL red-necked, 0.22 µm filter units (Nalgene), dispensed into the tissue culture tubes, and incubated at 37°C in 5% CO2 in air for at least 18h before use.

Lactate, Pyruvate, BSA in refrigerator
Pen/Strep in freezer
Other reagents in desiccator under vacuum
Place approximately 125 mL ultra-pure water into beaker with stir-pellet
Use sterile 2 mL pipette for Pen/Strep
Be careful not to create bubbles (stir too fast) when adding BSA
Carefully drop Pen/Strep onto BSA to facilitate dissolving

APPENDIX 2

TWO-DIMENSIONAL CHEMICAL STRUCTURES OF COMPOUNDS DESCRIBED IN INTRODUCTION

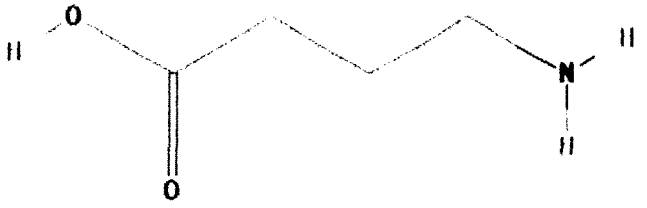

Fig. 20. GABA chemical structure. Gamma-aminobutyric acid $C_4H_9NO_2$ M.W. 103.11976 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=119, http://pubchem.ncbi.nlm.nih.gov/compound/119

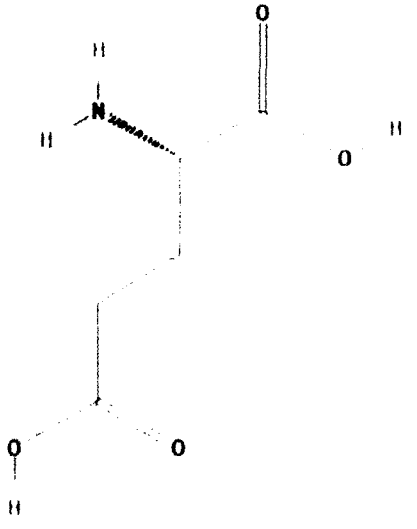

Fig. 21. Glutamate chemical structure. L-glutamic acid $C_5H_9NO_4$ M.W. 147.12926 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=33032, http://pubchem.ncbi.nlm.nih.gov/compound/33032

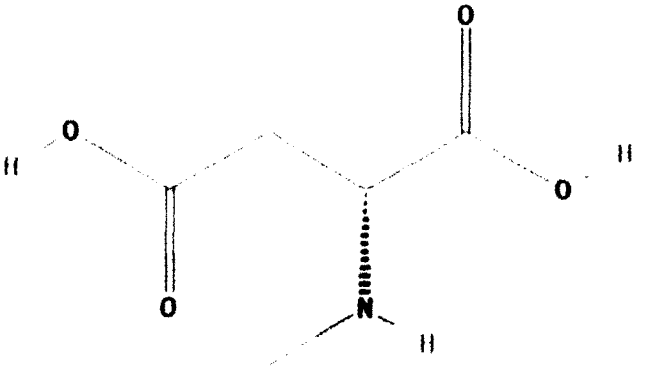

Fig. 22. NMDA chemical structure. *N*-methyl-D-aspartate $C_5H_9NO_4$ M.W. 147.12926 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=22880, http://pubchem.ncbi.nlm.nih.gov/compound/22880

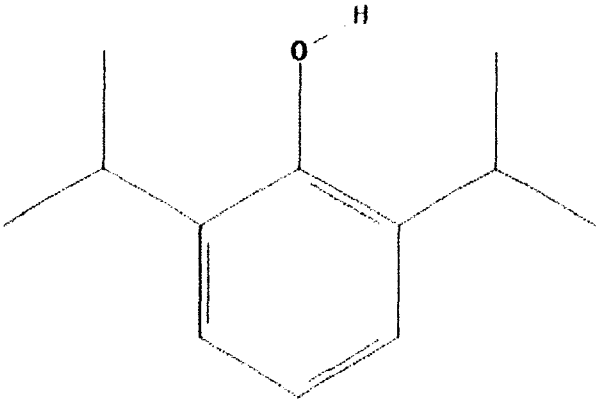

Fig. 23. Propofol chemical structure. 2,6-Diisopropylphenol $C_{12}H_{18}O$ M.W. 178.27072 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=4943, http://pubchem.ncbi.nlm.nih.gov/compound/4943

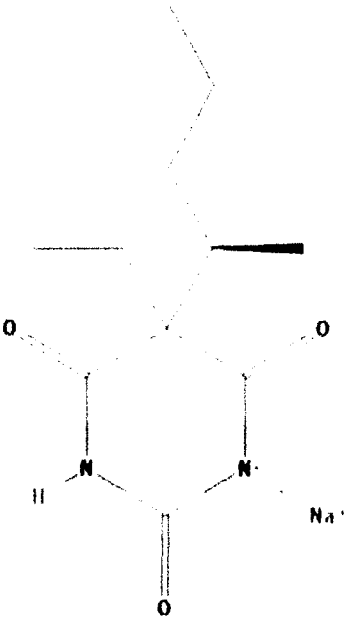

Fig. 24. Sodium pentobarbital chemical structure. Sodium;5-ethyl-5-[(2R)-pentan-2-yl]pyrimidin-3-ide-2,4,6-trione $C_{11}H_{17}N_2NaO_3$ M.W. 248.254049 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=23692753, http://pubchem.ncbi.nlm.nih.gov/compound/23692753

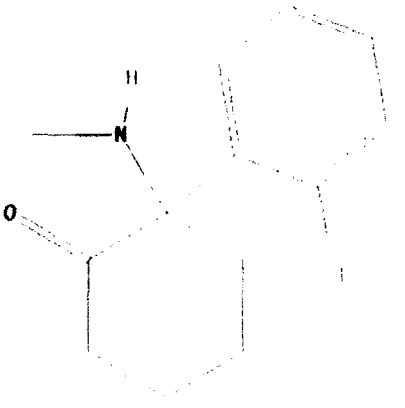

Fig. 25. Ketamine hydrochloride chemical structure. 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one;hydrochloride $C_{13}H_{17}Cl_2NO$ M.W. 274.18618 g/mol.[256] National Center for Biotechnology Information. PubChem Compound Database; CID=15851, http://pubchem.ncbi.nlm.nih.gov/compound/15851

VITA

Tyler Cameron Balak
Department of Biological Sciences
Old Dominion University
Norfolk, VA 23529

Education:

2015	Old Dominion University, Ph.D. candidate in Biomedical Sciences, graduation date: May 2015. Dissertation title: "The Effects of Propofol, Sodium Pentobarbital and Ketamine Hydrochloride on *In Vitro* Mouse Embryonic Development"
2004	University of North Carolina at Wilmington, B.S. Biology with Honors

Awards and Honors:

2004	Graduated Cum Laude; Awarded University Honors and Departmental Honors for Undergraduate Research in Biochemistry at UNCW
2003-2004	UNCW Honors Program Scholarship for Academic Excellence
2001	Inducted into Phi Eta Sigma National Honor Fraternity

Teaching Experience:

2013-2015	Adjunct Professor, South University. Anatomy and Physiology I, Anatomy and Physiology I Laboratory, and Medical Terminology
2005-2013	Graduate/Doctoral Teaching Assistant, Old Dominion University. Laboratory Instructor for Developmental Biology, Anatomy and Physiology I and II

Research Experience:

2007-2013	Graduate Research in Embryology Laboratory, Old Dominion University. Experimental techniques in production, microsurgical collection, in vitro culture, and manipulation of mouse embryos; quality control assays of IVF culture medium preparations, labware compounds, sterilization compounds, and methods.
2005-2006	Graduate Research at Frank Reidy Center for Bioelectrics at Old Dominion University. Worked to fabricate and calibrate pulse plunge apparatus designed to apply high voltage nanosecond pulses to cells suspended in solution then instantaneously flash freeze with liquid nitrogen.